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C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

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BACTERIAL INHIBITION

I. GERMICIDAL ACTION IN MILK

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From the Division of Dairy Bacteriology, University of Illinois

Received for publication April 5, 1920

Improvement of our milk supply and the evolution of better methods for its handling will be aided by a more detailed knowledge of the growth of bacteria in milk. The growth of bacteria in bouillon, as shown by Buchanan and others, follows a rather definite curve, a period of delayed multiplication or latency preceding the period of most rapid growth or logarithmic increase. In milk, however, plate counts have, in many cases, demonstrated a decrease of bacteria before the period of logarithmic increase. Whether or not this is a true germicidal action has been the subject of intermittent controversy ever since Nuttall and Buchner first reported a germicidal action in blood in 1888. Later workers, such as Rosenau, have given very complete historical reviews of the early literature, so that only a few points of conflict in ideas are mentioned below.

The earlier workers were sharply divided. Freudenreich, Heim, Hesse, Weigmann, and Kolle found that *Vibrio cholerae* was killed in raw milk, but Honigmann and Basenau observed no germicidal action on this organism. Schottelius reports a better growth of *Corynebact. diphtheriae* in raw milk than in sterilized milk or bouillon. Hunziker in 1901 is apparently the first and only investigator seriously to take account of the possible variation in germicidal power in the milk from different cows. He showed a marked decrease in bacteria in the milk of some cows and none in others, but observed only the action of the milk of individual cows on their own milk flora and used no pure cul-

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tures. Stocking explains the reduction in the number of bacteria in the mixed milk from a herd of thirty cows as due to the death of those organisms which find milk an unfavorable medium while a parallel increase of lactic acid forms is taking place; but he makes no comparison of the behavior of the first group in raw and heated milk to show that the decrease is not due to a property of raw milk only. The relationship between germicidal action and temperature has been demonstrated by Koning, Hunziker, and others. Pure culture work from 1905 to 1909 by Hippius, Coplans, Brudny, Evans and Cope, Heinemann, and Rosenau has shown a definite germicidal action in varying degrees on quite a number of different species of bacteria, but the possibility of variation in individual cows is practically ignored and their conclusions are based on the action of only one or two cows. Heinemann found agglutinins for certain bacteria in milk serum and concluded that they seemed to bear some relation to the germicidal action. On assembling the work of the different investigators, it appears that much of the variation in results is due to a lack of consideration, in each case, of one or more of the essential factors.

It is the endeavor of this experimental work to correlate the important factors, emphasizing the individuality of the cow and the specificity of action of the bacteria, and by direct microscopic examination of the milk which is being tested to follow the growth of the bacteria and observe any evidence of agglutination. The milk was obtained from eleven cows from the University of Illinois herd, selected because in previous experiments they had shown a low germ content in the udder. Three organisms were used for inoculation in pure culture: *Bact. coli*, *Bact. lactis-acidi*, and a brilliant red chromogen isolated from a creamery can and designated Culture R. Near the middle of the milking, about 1 pint of milk, in approximately equal portions from each quarter of the udder, was drawn with aseptic precautions into a small sterile container. One half, to be inoculated raw, was held at 37°C., while the other half was heated to between 85°C. and 90°C. for two minutes and then cooled to 37°C. Ninety-nine cubic centimeter portions of the raw and heated milk in 300 cc.

glass-stoppered bottles were used for inoculation, and equal samples were kept for controls.

The work of Coplans, Rahn, Penfold, Chesney, Salter, and others on the latent period of growth in freshly inoculated bouillon has demonstrated that the most favorable conditions for transferring a culture are during its period of logarithmic increase and between media of the same composition at the same temperature. Therefore a constant temperature of 37°C. was used

TABLE 1

Growth of bacteria in raw milk at 37°C.

	1	2	3	4	5	6	7	8
	OCTOBER 13	OCTOBER 19	NOVEMBER 3	NOVEMBER 14	NOVEMBER 21	DECEMBER 5	DECEMBER 9	DECEMBER 21
TIME	Milk from cow number							
	134 190 212	134 190 212	155 188 134 196	188 134 196	188 134 196	208 180 179	208 201 181	201 208
minutes	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*
0	5,100	200	460	1,230	2,290	825	220	390
15		97	500	1,430	2,520			
30		142	550	1,600	2,470	1,210	250	300
60	100	112	440	1,630	3,260	1,610	290	350
90		124	540	1,900	3,450	1,290	250	240
120		210	330	1,970	3,730	1,580	470	470
180	4,900			3,530	4,630		380	480
210						11,400		
240		23	50	9,360	5,750		6,660	4,690
300				14,700	15,700			

throughout the experimental work to give a more intense action over a shorter period of time of observation. The milk inoculations, 1 cc. portions from five-hour milk cultures at 37°C., were high compared to the germ content of the raw milk, thus minimizing the influence of the original udder flora upon the growth in the raw inoculated milk. Numerical determinations were averaged from triplicate plates of 1 per cent lactose agar, made essentially according to the standard methods of the American Public Health Association.

The experimental work was divided into two parts: first, the determination of the individual variation in germicidal action in the milk of the eleven selected cows; and second, the variation in action upon pure cultures in the milk of the cows showing the best germicidal action. Table 1 gives the results of the first part, the study of variation in individual cows. The data comprise eight trials in which the mixed milk from different groups of cows, as indicated by their herd numbers, was plated as one sample. The cows were arranged in groups to introduce organ-

TABLE 2

Growth of bacteria in milk, raw and inoculated with Bact. coli, at 37°C.

TIME	1			2			3		
	JANUARY 26			FEBRUARY 9			APRIL 20		
	Cow 212			Cow 212			Cow 155		
	Uninoculated control	Inoculated		Uninoculated control	Inoculated		Uninoculated control	Inoculated	
	Raw	Raw	Heated	Raw	Raw	Heated	Raw	Raw	Heated
minutes	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*
0	970	6,900	8,500	420	31,100	31,800	30	5,600	6,500
30	1,070	13,500	24,800	410	48,400	60,000	30	6,300	15,100
60	890	900	62,000	440	10,100	101,000	50	500	14,800
90	930	2,100	135,800	1,290	9,300	190,000	40	100	50,300
120	1,590	4,800	250,000	5,040	10,400	1,035,000	40	−100	121,800
180	8,360	14,700	5,400,000	21,180	134,000	I*	50	−100	486,000
240	42,300	95,900	12,000,000	69,700	625,000	I*	420	1,600	I*

* I = Innumerable.

isms foreign to each cow's milk into each sample—which proved to be essential in these cases for germicidal action—and to show by elimination which cows possessed the germicidal property. Only three of the eight samples (columns 1, 2, and 3) show a decrease in bacteria distinct enough to be attributed to a germicidal action; the others show only an increase in growth, or a variation within the limits of experimental error. The samples of milk which show a germicidal action (columns 1, 2, and 3) are from six cows, numbers 134, 155, 188, 190, 196, and 212.

Three of these cows, numbers 134, 188, and 196, when tested together twice (columns 4 and 5) show only an increase in growth; hence the decrease in the first three columns is attributed to the three cows, numbers 155, 190, and 212. The milk of the other five cows, numbers 179, 180, 181, 201, and 208, when used in three different combinations, shows no evidence of a germicidal property. Of the eleven cows considered in table 1, only three possess a germicidal power. The individual cow therefore is seen to be an important factor in the study of bactericidal prop-

TABLE 3

Growth of bacteria in milk, raw and inoculated with culture R, at 37°C.

TIME	1			2		
	FEBRUARY 20			APRIL 3		
	Cow 212			Cow 155		
	Uninoculated control	Inoculated		Uninoculated control	Inoculated	
	Raw	Raw	Heated	Raw	Raw	Heated
minutes	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*
0	270	162,500	195,000	40	27,300	26,500
30	310	176,000	242,000	60	28,700	38,200
60	420	192,000	360,000	90	24,700	53,400
90	620	342,000	910,000	120	42,400	142,300
120	5,890	1,190,000	1,600,000	190	176,400	491,000
180	27,900	5,000,000	7,150,000	130	930,000	1,260,000
240	72,000	I*	I*	210	3,000,000	7,000,000

* I = Innumerable.

erties in milk, especially if this ratio of 3 to 11 is taken as an indication of the prevalence of these properties among individual cows.

To demonstrate the specificity of the germicidal property for different bacteria, pure cultures were inoculated into the milk of individual cows. From the results reported in table 1, cows 155 and 212 were selected for inoculation tests with pure cultures of *Bact. coli*, *Bact. lactis-acidi*, and Culture R, according to the technique reported above. The growth of the different organisms is recorded in tables 2, 3, and 4, which show the comparable

growth in the uninoculated raw milk control and in the two inoculated samples, raw and heated, which were identical except for the heating. The heated controls are not tabulated, for in every case only a few surviving spore-formers developed during the four-hour period of observation.

The growth of *Bact. coli* in the milk of these two cows corroborates the presence of a bactericidal property in their raw milk which is not evident in the corresponding heated sample. A comparison of the raw and heated milk samples inoculated

TABLE 4

Growth of bacteria in milk, raw and inoculated with Bact. lactis-acidi, at 37°C.

TIME	1			2		
	MARCH 20			APRIL 20		
	Cow 212			Cow 155		
	Uninoculated control	Inoculated		Uninoculated control	Inoculated	
	Raw	Raw	Heated	Raw	Raw	Heated
minutes	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*	*per cc.*
0	170	17,500	15,700	30	111,700	119,300
30	140	44,500	34,600	30	265,000	251,000
60	110	93,000	80,400	50	295,000	405,000
90	680	166,000	136,000	40	677,000	939,000
120	2,830	416,000	394,000	40	2,200,000	2,550,000
180	11,900	1,800,000	1,250,000	50	I*	I*
240	124,000	I*	I*	420	I*	I*

* I = Innumerable.

with this organism (table 2), shows in every case a marked reduction of numbers of bacteria in raw milk and a rapid increase in numbers in the heated milk. The action is positive, for it is practically the same in two separate tests with cow 212, and is more intense with cow 155, for the count went below the 100 dilution at 120 and 180 minutes. In the milk of cow 212 there is a return of the growth to the normal logarithmic rate of multiplication, after 120 minutes. This germicidal action, as compared to the growth in the heated milk, is illustrated more clearly in chart 1, in which the curves are plotted from the logarithms of the averages of the raw and heated milk samples from table 2.

Culture R (table 3) grows more slowly for the first 90 minutes in the raw milk than in the heated milk. The raw milk growth is distinctly different from that of *Bact. coli*, however, for only a

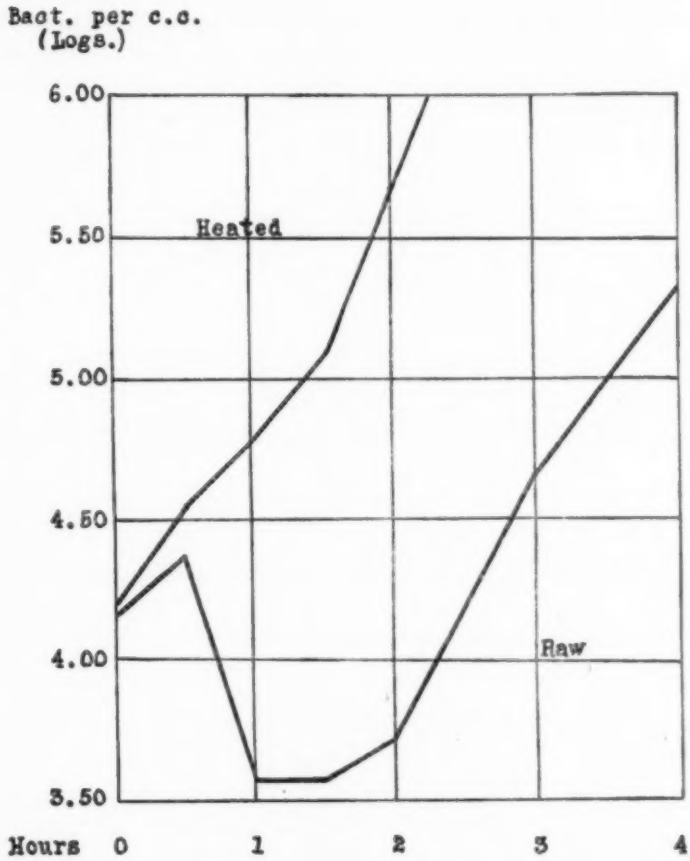

CHART 1. GROWTH OF BACTERIA IN MILK INOCULATED WITH *B. coli* AT 37°C.

lag phase and no germicidal action is evident. This is shown in chart 2, which is based on the logarithms of the averages of table 3. It is noticeable that a type of growth similar to that repre-

sented by the raw milk curve of chart 2, i.e., a growth in which there is no decrease in numbers but a definite lag phase preceding the logarithmic increase, is found in the majority of the uninoculated raw milk samples (table 1, columns 4, 5, 6, 7, and 8, and all the uninoculated controls of tables 2, 3, and 4). Consider-

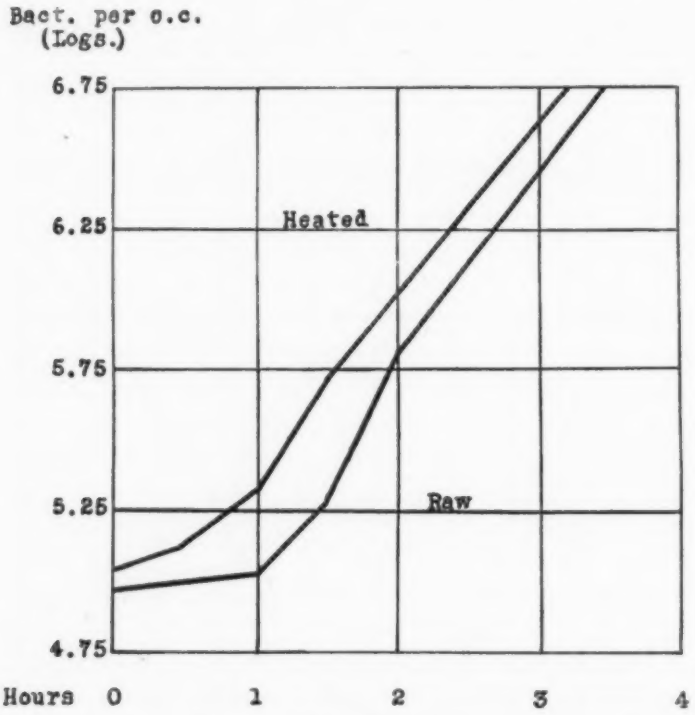

CHART 2. GROWTH OF BACTERIA IN MILK INOCULATED WITH CULTURE R AT 37°C.

ing the number of different species of bacteria represented in the uninoculated raw milk samples, this would seem to be a more common type of growth in raw milk than the germicidal type.

Table 4 shows practically no difference in the rate of multiplication of *Bact. lactis-acidi* in the raw milk and in the heated

milk. Applying the formula of Buchner, Longard, and Riedlin, the average generation time for the period from inoculation to 120 minutes is 26.9 minutes in the raw milk and 26.4 minutes in the heated milk, a difference of only 0.5 minute. In the raw milk of these two germicidal cows there is no bactericidal action on *Bact. lactis-acidi*, and apparently no retarding influence, such as is found for Culture R and the organisms in the uninoculated

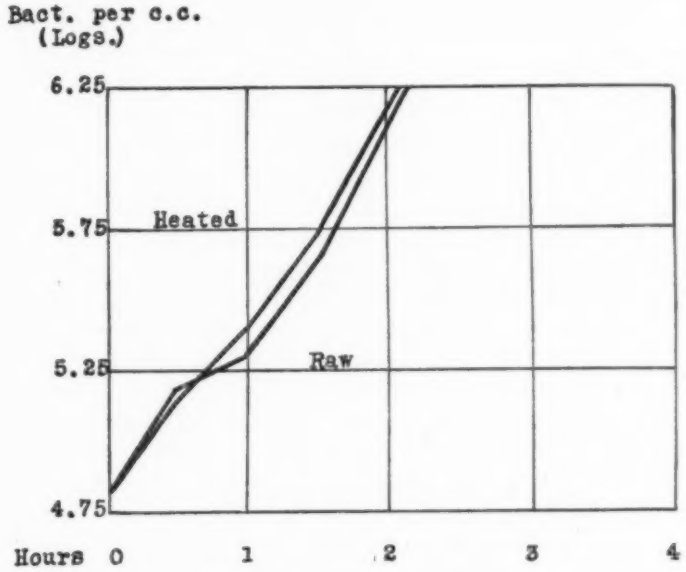

CHART 3. GROWTH OF BACTERIA IN MILK INOCULATED WITH *Bact. lactis-acidi* AT 37°C.

controls. The similarity in the growth curves of the raw and heated milk is shown in chart 3, plotted from the logarithms of the averages from table 4.

This specificity of the germicidal action for the organism is best illustrated by chart 4, in which are assembled the raw milk curves from charts 1, 2, and 3. It is of interest to note that throughout tables 2, 3, and 4 the uninoculated raw milk controls of these two cows, numbers 155 and 212, show no germicidal action

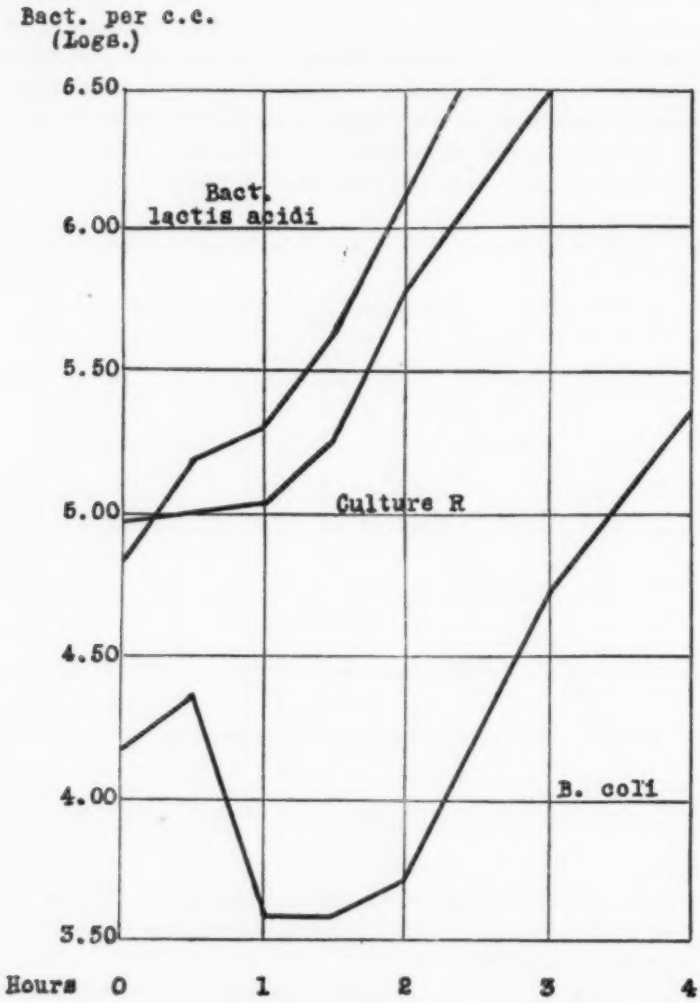

CHART 4. GROWTH OF BACTERIA IN RAW MILK AT 37°C.

toward their own udder organisms, while in table 1 (columns 1, 2, and 3) their milk shows distinct bactericidal properties for the mixed flora of several cows. This also emphasizes the specificity of the germicidal property and suggests an immunity of the organisms constantly present in the cow's own udder.

Observations were made on agglutination in connection with the numerical determination of the bacteria by plate count. The microscopical examinations for agglutination were made according to Breed's method for direct count from the milk cultures just before inoculation, and from the raw and heated milk just after sampling for plating. There was in no case agglutination in the heated milk or in the milk cultures used for the inoculation, which were made from sterilized milk. In the raw milk there was a very well-defined agglutination of Culture R, which appeared in large clumps of from twenty to several hundred, as opposed to an even distribution of individual organisms throughout the preparations made from the comparable heated milk. *Bact. lactis-acidi* showed a similar but weaker action in the raw milk, forming clumps of from four to twenty organisms. The preparations with *Bact. coli* in the raw milk were not so satisfactory, for where the counts were low the scarcity of organisms made microscopical enumeration difficult. A few small clumps were noted in the raw milk smears made at 240 minutes on January 26 and February 9 (table 2, columns 1 and 2).

Several theories have been advanced as explanations of germicidal action. Its specificity and reaction to heat suggest a serologic origin. The presence of agglutinins, antitoxins, hemolysins, opsonins, and other antibodies in raw milk has been frequently reported since Ehrlich's work in 1893. The decrease in bacteria in raw milk has been explained by some investigators as an apparent one, due to agglutination, rather than as a true bactericidal action, but the observations reported above do not show a correlation of agglutination and germicidal action. Summarizing the experimental data, Culture R gives the best agglutination but no germicidal action; *Bact. lactis-acidi* gives a good agglutination and an immediate increase in growth; and *Bact. coli* shows

much weaker agglutination than Culture R and a strong germicidal reaction. If the decrease was only apparent, owing to agglutination, one would expect to find by microscopic count in the comparable raw and heated milk approximately equal numbers of individual bacteria clumped in the raw milk and evenly distributed in the heated milk preparation. Such is not the case. The shaking of the samples and dilutions was uniform and vigorous (thirty times) and a comparison of the plate count and the ratio of clumps in the raw milk to individuals in the heated milk would indicate that the clumps were broken up. For example, a typical case with Culture R showed a plate count ratio of 46 to 100 (raw to heated), and the corresponding stained preparation showed a ratio of 1 to 100 (clumps to individuals). With *Bact. coli* (table 2, column 2) the microscopic count and plate count check very closely The microscopic examination at 120 minutes showed three small clumps in the raw milk in the entire preparation of 0.01 cc., or approximately 7500 bacteria per cubic centimeter, and in the heated milk preparation an average of 1.5 bacteria per microscopic field, or approximately 1,500,000 bacteria per cubic centimeter. This compares very favorably with the plate count of 10,400 per cubic centimeter in the raw milk and 1,035,000 per cubic centimeter in the heated milk (table 2, column 2, 120 minutes). Evidently there is an actual decrease of bacteria with *Bact. coli* as shown by the plate count and the microscope. It is of interest to note the similarity in germicidal action in the growth curves of *Bact. coli* in raw milk (chart 1) and *Bact. coli* in normal rabbit-serum (Chick).

In the freshly drawn mixed milk from a herd of cows the action of bacterial inhibition is variable because of its specificity. The numbers of species of organisms increase as contaminations from the cows, barn, utensils, etc., are added to the original udder flora. Where only the total number of bacteria in the milk is considered, a decrease in numbers may be evident, or one unaffected strain by its rapid increase may completely hide the germicidal action on another less numerous strain, particularly if the action is caused by only a few cows. The predominance of the lactic acid organisms with the increasing age of the milk might be

attributed to bacterial inhibition, for the lactic acid organisms are apparently unaffected by it, increasing immediately from the start, whereas the other organisms seem to be restrained in growth and, in some cases, decreased in numbers.

SUMMARY

1. The combined evidence of microscopic examination and plate count demonstrates a germicidal property, or actual decrease in numbers of bacteria in raw milk under certain conditions.

2. The germicidal property is destroyed by heating to between 80° and 90°C. for two minutes.

3. The germicidal action is specific, depending on both the individual cow and the species of bacteria.

4. No common relation between agglutination and bacterial inhibition is noted, except that both are destroyed by heating the milk.

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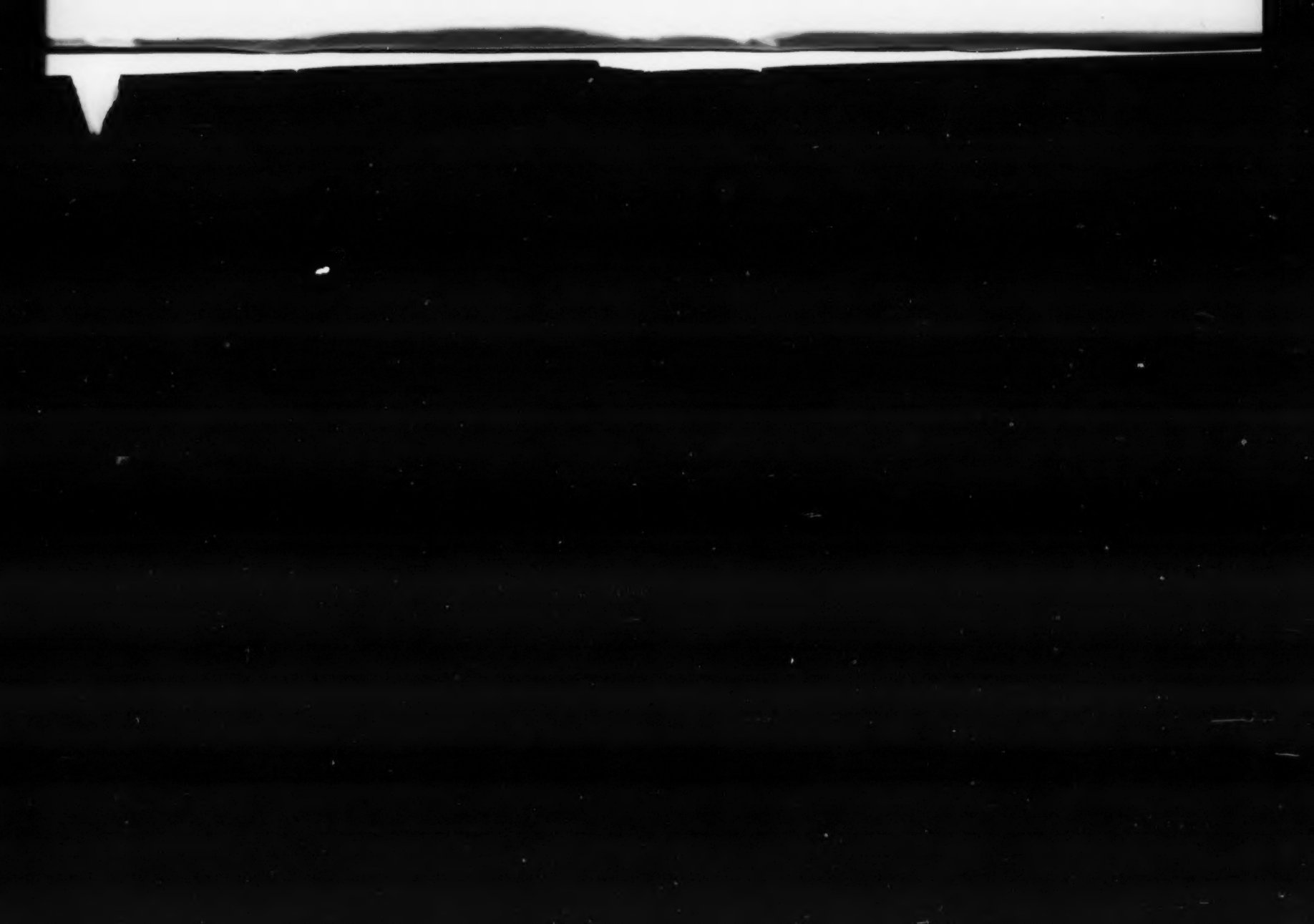

BACTERIAL GROUPS IN DECOMPOSING SALMON

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In a previous paper (Hunter, 1920) regarding the decomposition of salmon the total counts of bacteria found in the muscular tissue of the back and belly of the fish were given. The occurrence of these bacteria in the viscera and various organs of the salmon in different stages of decomposition was also discussed. The data presented were all obtained from experiments upon salmon handling as conducted in the Puget Sound region. From the mixed cultures and the plates made in the field 300 cultures were isolated and studied with the idea of grouping or typing the organisms significant in the decomposition of the salmon. Special care was taken to select those colonies which predominated on the plates.

It was evident that many organisms were obtained which played no part in the decomposition of the fish and that there were also many duplicates among the original 300 cultures. In order to eliminate the organisms which did not decompose salmon, and to check off the duplicates, use was made of a specially prepared fish broth.[2] The 300 cultures were grown in this

[1] This work was done under the supervision of Dr. Charles Thom to whom I am indebted for criticism and suggestions.

[2] This medium was prepared in the following manner by Mr. B. A. Linden of this laboratory:

To 1000 grams of finely chopped saltwater trout, or weakfish, from which the skin and bones had been removed, was added 1000 cc. of distilled water and 15 grams of peptone. The infusion was made by heating in the Arnold sterilizer or on a water bath at 95° to 100°C. for one hour with occasional stirrings. The juice was strained through cheese cloth with a meat press, filtered through cotton and the reaction adjusted to neutral. The infusion was then heated in the Arnold for thirty minutes at 100°C. and filtered, using folded filter papers. About 10 cc. was placed in each tube with about 1.5 grams of raw fish. For anaerobic cultures the surface was covered with a layer of liquid petrolatum. The medium was sterilized in the autoclave for fifteen minutes at 15 pounds.

medium, aerobically and anaerobically, for one week at 30°C. They were examined daily for the evolution of foul odors and for any digestion of the fish in the bottom of the tube. At the end of one week each culture was tested for indol production with paradimethylamidobenzaldehyde and hydrochloric acid. Only those cultures which produced indol, foul odors, or both, in this medium were saved for further study. In this manner the original number of cultures was reduced to 65 and these organisms were then studied in more detail in order to check the duplicates. The author, however, is aware that, while the discarded organisms had no putrefactive action in pure culture, they may assist in the decomposition of the salmon when associated with the bacteria which have been studied. In spite of this fact the present study has been limited to those cultures producing indol or foul odors when grown in pure culture.

On the basis of their morphology and their reactions in litmus milk, gelatin, potato, tryptophane broth and glucose, lactose and sucrose broths these 65 cultures were compared and duplicates eliminated so as to reduce the number to 43. Although some of these 43 cultures were very similar in many respects they all varied sufficiently to be considered different organisms and each of the 43 was studied and identified.

Of the 235 cultures rejected as playing no part in the decomposition of the salmon 4 were yeasts, 16 micrococci, 43 streptococci and 172 bacilli of varying morphology. All of the organisms regarded as significant in the decomposition of the fish are bacilli. Six of the cultures are pigment producers while the remaining 37 produce no pigment. The cultural reactions of these 43 organisms are summarized in tables 1, 2, and 3. For convenience the results are given in three separate tables, table 1 being the reactions of those organisms, exclusive of the pigment producers, which liquefy gelatin, table 2, those which do not produce pigment and do not liquefy gelatin and table 3, those which produce pigment.

In studying these bacteria but little attempt has been made to identify any of them, except the pigment producers, as specific organisms. It may be that an extended series of tests in various

carbohydrate media would identify as species or strains those forms which ferment the sugars but for the purposes of this investigation it has been considered sufficient simply to determine the group to which they belong. The studies of Winslow, Kligler and Rothberg (1919) on the classification of the colon-typhoid group and the work of Levine (1918) on the classification of the colon-cloacae group make it possible to identify those bacteria which produce acid, gas, or both, in glucose and lactose. On the other hand the inadequate descriptions in the literature of the non-fermenting bacteria from soil and water made it very difficult to identify unknown organisms of this kind.

In table 1 are tabulated the cultural reactions of 20 organisms which liquefy gelatin. Of these 13 have been identified as resembling *Bact. cloacae* (Jordan, 1890) in that they are small, Gram negative, gelatin-liquefying bacilli which produce acid and gas in glucose, lactose and sucrose, give a positive Voges-Proskauer and a negative methyl red reaction. Eight of these 13 cultures differ from the type in the production of indol.

Of the remaining 7 cultures described in table 1, 3 are very similar to *Bact. formosum* as described by Ravenel (1896) while the other 4 resemble nothing described in the literature and evidently belong in a group of unidentified water and soil bacteria.

The reactions given in table 2 show that of these 17 cultures 3 resemble *Bact. coli*, 2 *Bact. communior* and 3 *Bact. aerogenes*. The reaction in litmus milk in each case is not always characteristic but the reactions in glucose, lactose and sucrose broths and the results of the methyl red and Voges Proskauer tests would place them in the groups indicated. Culture 70 closely resembles *Bact. alcaligenes*. The remaining 8 cultures in this table somewhat resemble a number of organisms described by Chester (1901) in a group of motile, Gram-negative, non-liquefying bacteria from soil and water which form no spores and do not ferment any of the carbohydrates. The original descriptions of these bacteria, however, are inadequate for identification of the organisms studied. Until further work is done on this group, therefore, they cannot be designated other than as belonging to a large group of unidentified bacteria from the water and soil.

TABLE 1

Cultural reactions of gelatin-liquefying bacteria from decomposed salmon

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIO-CHEMICAL REACTIONS							TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges Proskauer	
110	Gills and intestines	Small bacilli	0	Peptonized	0	+	0[x]	0	0	0	0	
137	Mouth	Medium size bacilli	0	Peptonized	0	+	0	0	0	0	0	
139	Mouth	Small bacilli	0	No change	0	0	0	0	0	0	0	
184	Kidney	Small bacilli	0	Decolorized	0	+	0	0	0	0	0	*Bact. formosum* (Ravenel)
192	Gills and flesh of belly	Small bacilli	0	Decolorized	0	+	0	0	0	0	0	*Bact. formosum* (Ravenel)
168a	Gills	Small bacilli	0	Decolorized	0	+	0	0	0	0	0	*Bact. formosum* (Ravenel)
103a	Mouth and intestines	Small bacilli	0	Peptonized	+	+	A[y]	0	0	0	0	
70a	Flesh of belly	Short, thick bacilli	0	Coagulated	+	+	AG[z]	AG	AG	0	+	*Bact. cloacae*
118	Gills	Short, thick bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. cloacae*
130a	Mouth	Short, thick bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. cloacae*
138	Mouth and gills	Small bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. cloacae*
144	Gills and stomach	Short, thick bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. cloacae*
156	Gills and ceca	Short, thick bacilli	0	Coagulated	+	+	AG	AG	AG	0	+	*Bact. cloacae*
205a	Flesh	Medium size bacilli	0	Acid	+	+	AG	AG	AG	?	+	*Bact. cloacae*
235	Flesh of back	Medium size bacilli	0	Acid	+	+	AG	AG	AG	0	+	*Bact. cloacae*

253a	Flesh of back	Medium size bacilli	0	Acid	+	+	AG	AG	AG	?	+	*Bact. cloacae*
262	Flesh of belly	Medium size bacilli	0	Acid	+	+	AG	AG	AG	0	+	*Bact. cloacae*
273	Flesh of back	Medium size bacilli	0	Acid	0	+	AG	AG	AG	0	+	*Bact. cloacae*
292	Flesh of belly	Medium size bacilli	0	Acid	+	+	AG	AG	AG	0	+	*Bact. cloacae*
1	Ceca	Short, thick bacilli	0	Coagulated	+	+	AG	AG	AG	0	+	*Bact. cloacae*

[x]—0 indicates neither gas nor acid produced.
[y]—A indicates acid but no gas produced.
[z]—AG indicates acid and gas.

TABLE 2

Cultural reactions of non-liquefying bacteria from decomposed salmon.

CULTURE	SOURCE	SHAPE	GRAM STAIN	LITMUS MILK	BIO-CHEMICAL REACTIONS							TYPE RESEMBLED
					Indol	Nitrate reduced	Glucose	Lactose	Sucrose	Methyl red	Voges Proskauer	
F	Intestine	Small bacilli	0	Decolorized	0	+	0[x]	0	0	0	0	
76	Flesh of belly	Small bacilli	0	Peptonized	0	+	0	0	0	0	0	
92a	Gills	Small bacilli	0	Decolorized	+	+	0	0	0	0	0	
108	Blood	Short, thick bacilli	0	No change	0	+	0	0	0	0	0	
125	Gills	Slender bacilli	0	No change	+	+	0	0	0	0	0	
125a	Gills	Small bacilli	0	No change	+	+	0	0	0	0	0	
190	Mouth	Medium size bacilli	0	Decolorized	0	+	0	0	0	0	0	
291	Flesh of belly	Medium size bacilli	0	Decolorized	0	+	0	0	0	0	0	
70	Flesh of belly	Small bacilli	0	Alkaline	0	+	0	0	0	0	0	*Bact. alcaligenes*
200	Flesh of back	Medium size bacilli	0	Coagulated	+	+	AG[z]	AG	0	+	0	*Bact. coli*
203	Flesh of back	Medium size bacilli	0	Acid	0	+	AG	AG	0	+	0	*Bact. coli*
285	Flesh of back	Medium size bacilli	0	Coagulated	+	+	AG	AG	0	+	0	*Bact. coli*
261	Flesh of back	Medium size bacilli	0	Coagulated	+	+	AG	AG	AG	+	0	*Bact. communior*
268	Flesh of back	Medium size bacilli	0	Coagulated	+	+	AG	AG	AG	+	0	*Bact. communior*
265b	Flesh of back	Medium size bacilli	0	Decolorized	+	+	AG	AG	AG	0	+	*Bact. aerogenes*
259	Flesh of back	Medium size bacilli	0	Acid	0	+	AG	AG	AG	0	+	*Bact. aerogenes*
95	Gills	Medium size bacilli	0	Coagulated	0	+	AG	AG	AG	0	+	*Bact. aerogenes*

[x]—0 indicates neither acid nor gas.

[z]—AG indicates both acid and gas.

TABLE 3

Cultural reactions of pigment producing bacteria from decomposing salmon

CULTURE	SOURCE	SHAPE	PIGMENT	GRAM STAIN	LITMUS MILK	BIO-CHEMICAL REACTIONS							ORGANISM RESEMBLED
						Indol	Gelatin liquefied	Nitrates reduced	Glucose	Lactose	Sucrose	Motility	
C	Intestine	Small bacilli	Green	0	Peptonized	±	+	0	A[y]	0	0	+	?
216	Flesh of back and belly	Small bacilli	Green	0	Peptonized	0	+	0	A	0	0	+	?
14a	Slime and belly	Short, thick bacilli	Orange	±	No change	0	0	0	0[x]	0	0	0	*Bact. breve* (Frankland)
113b	Stomach	Medium size bacilli	Orange	0	Peptonized	+	+	+	A	0	0	0	*Bact. caudatum* (Wright)
180	Stomach	Medium size bacilli	Orange	+	No change	0	0	+	0	0	0	+	*Bact. aurantiacum* (Frankland)
124	Ceca	Slender bacilli	Flesh-colored	0	Peptonized	+	+	0	A	0	0	+	*Erythrob. carnicolor* (Frankland)

[x]—0 indicates neither acid nor gas produced.

[y]—A indicates acid but no gas.

Of the 6 cultures producing pigment on gelatin and agar 3 produce an orange pigment, 1 a flesh-colored pigment and 2 a greenish pigment. Cultures 14a, 113b and 180, producing orange pigment, have been found to resemble closely *Bact. breve* (Frankland, 1894), *Bact. caudatum* (Wright, 1895) and *Bact. aurantiacum* (Frankland, 1894) respectively. The organism producing a flesh-colored pigment, 124, is very similar to *Erythrobacillus carnicolor* (Frankland, 1894). The original descriptions of these bacteria are not adequate for positive identification but the reactions of the organisms studied have been found to check with the limited descriptions given in the literature. The two cultures producing green pigment, C and 216, do not closely resemble any bacteria described. These two organisms are undoubtedly identical and might well have been considered as a single culture. All cultures of C and 216 have an aromatic odor.

No strict anaerobes and no spore-forming bacteria were isolated from the decomposed salmon. The absence of bacteria of the mesentericus group was surprising in view of the fact that during 1919 an examination of 530 cans of salmon packed in Alaska showed 42 per cent of the cans to contain living bacteria of this group (Hunter and Thom, 1919). No such organisms, however, have been isolated from the salmon caught and packed in the Puget Sound region. In considering the source of the organisms found in the uncooked fish it is evident that neither strict anaerobes nor spore-forming bacteria should be expected. As shown in the previous paper on the subject (Hunter, 1920) the digestive tract and viscera of the salmon are sterile when there is no food in the tract and when the fish are examined immediately after they are caught. The presence of bacteria in the gills, mouth and on the skin at all times and the late appearance of these organisms in the digestive tract and in the viscera indicate that the infection after death is from the outside inward rather than from the inside outward as is ordinarily found in the case of warm-blooded animals. We would expect the bacteria in the gills and mouth and on the skin to be those forms, the natural habitat of which is the sea-water in which the salmon live.

Identification of these 43 cultures has shown that this is actually the case. Most of the bacteria isolated from the decomposed salmon were originally described as water or sewage organisms, although a few were originally isolated from the soil. While these facts do not absolutely preclude the possibility of strict anaerobes and spore-formers being present, actual examination of 300 cultures from a large number of salmon in various stages of decomposition has shown that the flora of decomposed salmon is that of the sea-water from which they were taken. Furthermore, there was apparently no contamination with spore-forming organisms in the boats or in the cannery subsequent to the catching of the fish. The bacteria isolated from fish held for four days in the cannery are the same forms which were isolated from the gills and mouth when the salmon were examined immediately after being caught.

The bacteria responsible for the decomposition of the fish are not, in the strict sense, putrefactive and there is no rapid digestion of the flesh of the fish in the cultures. Each culture examined, however, did give some sort of "off" or foul odor in the fish medium and many showed the character of indol production when grown in this medium. It was noted that when grown in a beef infusion broth, with or without a small amount of carbohydrate added, no odors suggestive of decomposition were obtained. This makes it apparent that the flesh of fish is more susceptible to decomposition by ordinary water bacteria than is the flesh of the higher animals.

Although the salmon, when not feeding, has no bacteria in its intestines which may play a part in its subsequent decomposition, the accumulation of ordinary water bacteria in the gills and mouth and on the skin provides a flora which brings about a slow decomposition of the body of the fish after death.

SUMMARY

In a study of the bacteria which decompose raw salmon, 43 cultures were isolated as significant. On identification 21 cultures were found to be members of the colon-cloacae group. One culture resembled *Bact. alcaligenes* and 3 cultures resembled

Bact. formosum. Six cultures produced pigment. Twelve cultures not identified to type, evidently belong to a large group of water and soil bacteria which have not been adequately studied.

The bacteria isolated from decomposing salmon were found to be those which are described in the literature as water, sewage and soil organisms.

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THE INFLUENCE OF VARIOUS CHEMICAL AND PHYSICAL AGENCIES UPON BACILLUS BOTULINUS AND ITS SPORES

I. RESISTANCE TO SALT

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A number of statements are made in various text books concerning the vitality and longevity of *Bacillus botulinus* and its spores, as influenced by various chemical and physical agencies. Having a number of different strains of *B. botulinus* in pure culture, it seemed desirable that these statements should be evaluated in the light of past personal experiences with various strains of this bacillus. A study of the various agencies has been taken up in the order of their importance as nearly as practicable but from the nature of the problems it has been possible to conclude some of the less important phases first. For this reason the studies of the resistance of *B. botulinus* and its spores to salt are presented at this time. The other data will be published in the order of their completion.

The statement is made in Kendall's "General Pathological and Intestinal Bacteriology," that pickling in 10 per cent salt solution is destructive to the spores of *B. botulinus*, killing them within a week, and in Herzog's "Disease-Producing Microorganisms," 5 or 6 per cent salt is said to prevent the multiplication of the bacillus.

Nineteen cultures of *B. botulinus* were used in the tests. Some of these strains may be identical, as a full history was not sent by some of the laboratories from which they were obtained.

The medium used was glucose pork gelatin-broth having a reaction of -0.5, and the percentages of salt used were from

one to ten inclusive, the solutions being made by adding sufficient broth (specific gravity taken roughly as 1.00) to 1, 2, 3, etc., grams of salt to make 100 cc. Each separate concentration of broth was inoculated heavily with *B. botulinus* so that the resistant minority would survive. A layer of sterile paraffin oil about 1 to 1.5 cm. deep was placed on the broth cultures, which were cultivated at room temperature. Examinations, macroscopic, and microscopic when necessary, were made every twenty-four hours for ten or eleven days with the first five strains and for seven days only with the remainder. Where tubes showed no growth, transfers were made from the tube of next lower concentration showing growth. Growth is shown first by cloudiness, then generally by gas production (more or less vigorous), followed by a precipitate, more or less heavy. Where growth did not show by these signs transfers were made into similar broth containing only 0.5 per cent salt and incubated. Examinations were made of the 10 per cent salt broth cultures only, after considerable periods of time as follows:

	days
Strains GO6, Mul 433, Alb 175, Alb 175B, Chi 595, C91 and GS	27
Strain Columb 31	28
Strains Mul 435, Mul 471, and AM of NH	31
Strains NBS.,[6] Columb 1 and N. Y. City B. of H.	32
Strains Cal I, Cal II, Cal III, Cal IV and Cal VI	49

Table 1 indicates the growth in salt broth for the nineteen strains used. The occasional strains showing no growth at various times and salt concentrations are indicated.

It will be noted by examining the above table that only two out of the total of nineteen strains failed to show growth at any percentage of salt from 1 to 5 within the seven day period, and further that these two strains were both positive at the end of 27 days.

Table 2 indicates the record of the individual strains in the different salt broths. In this table the + sign signifies positive growth throughout the whole period of time unless otherwise indicated. It will be noted that ten out of the nineteen strains showed growth throughout the whole period.

Strains Cal I, Cal II, Chi 595, Mul 435, and AM of NH, are from the same original source and are of low virulence, and the results are practically the same, i.e., growth occurred throughout in all percentages of salt, and their vitality was not apparently impaired by a sojourn for 49, 27 or 31 days respectively in 10

TABLE 1

Resistance of B. botulinus

AGE	PER CENT SALT					
	1	2	3	4	5	10
days						
1	+	+°	+°	+	+	
2	+	+	+	+	+	
3	+	+	+	+	+	
4	+	+	+	+	+	
5	+	+	+	+	+	
6	+	+	+	+	+	
7	+°°°	+°°°	+°	+°°	+°°°	
8*	+	+	+	+	+	
9*	+	+	+	+	+	
10**	+	+	+	+	+	
11*	+	+	+	+	+	
27						+
28						+
31						+°°°°
32						+
49						+***

° Alb 175 negative.
°° Alb 175 and Alb 175B both negative.
°°° Alb 175B negative.
°°°° Strain Mul 471 doubtfully positive at this time.
* Strains Cal II, Cal III, Cal IV, and Cal VI only, examined.
** Strain Cal I only, examined.
*** Strain Cal III, only, negative.

per cent salt broth. These results are not confined to strains of low virulence, however. Similar results were obtained with strains of high virulence such as Cal IV, NBS, GO6, Mul 433 and Alb 175B, which survived 10 per cent salt for 49, 32, 27, and 27 days respectively. Cal III, Cal VI, and Mul 471, virulent strains, were the only strains virulent or non-virulent showing

TABLE 2

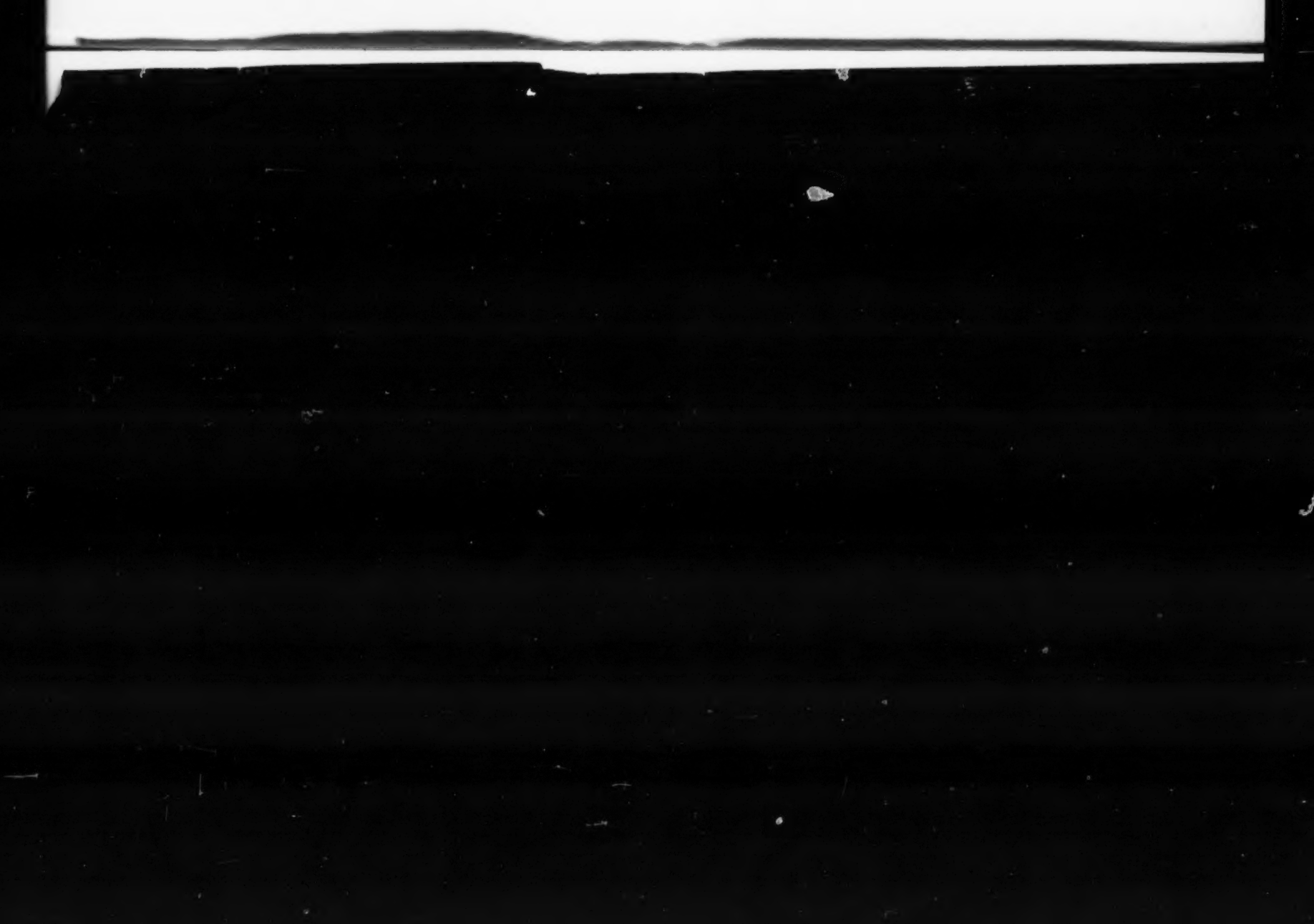
Resistance of individual strains of B. botulinus

STRAIN	PER CENT SALT					REMARKS
	6	7	8	9	10	
Cal. I	+	+	+	+*	+*	* These were negative the eighth and ninth days
Cal II	−*	−**	−**	−***	−****	* Positive after the first day ** Positive after the second day *** Positive after the second day, negative the third day and fourth days, then positive throughout **** Positive after the third day
Cal III	+	+	+	+	+*	* Negative the forty-ninth day
Cal IV	+	+	+	+*	+**	* Negative after the sixth day ** Negative the eleventh but positive the forty-ninth day
Cal VI	+	+	+	+	+*	* Negative the forty-ninth day
NBS	+	+	+	+	+	
Columb. 1	+	+	+	+	+	
GO6	+	+	+	+	+	
Columb. 31	+	+	+	+	+	
New York City Board of Health	+	+	+	+	+	
Mul 435	+	+	+	+	+	
Mul 471	+	+	+	+	+*	* Growth questionable on thirty-first day
Mul 433	+	+	+	+	+	
Alb 175	+	−*	−*	−**	−*	* Positive after the first day ** Negative after sixth and seventh days
Alb 175B	+*	+	+**	+*	+	* Negative after the fifth day ** Negative after the sixth day
Chi 595	+	+	+	+	+	
AM of NH	+	+	+	+	+	
C91	+	+	+	+	+	
GS	+*	+*	+	+*	+*	* Growth negative on the seventh day. In 10 per cent salt growth was positive however at the twenty-seventh day

growth at 11 days yet apparently not surviving 10 per cent salt at the end of 49 or even 31 days. All the other strains with two exceptions gave good growth every day for seven or eleven days after inoculation, and in addition, from 27 to 49 days after inoculation, in 10 per cent salt broth. Quantitative results were not obtained except in that a loop transfer from the 10 per cent salt broth culture gave a definite and decided clouding in the alkaline glucose pork gelatin-broth in 24 hours in every case as stated, with but two or possibly three exceptions. These results were checked microscopically.

These data prove quite conclusively that a large number of strains of *B. botulinus* are not inhibited by percentages of salt ranging from 1 to 10, when growing in a medium of an alkalinity of −0.5. This may not signify that *B. botulinus* will survive 10 per cent or less salt used in pickling, as the additional factor of acidity enters here, and this anaerobe is said to be injured—some strains quite decidedly so—by even a slightly acid medium (+0.5 to +0.8). However, the results obtained above seem to indicate strongly that it is not the salt which is the inhibiting factor in the destruction of *B. botulinus* and its spores by pickling solutions.

The data for the above tables were worked out by Miss Ruth Normington, a post-graduate student.



TIME SAVING BACTERIOLOGICAL APPARATUS

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In bacteriological laboratories the preparation of media and the cleaning of used apparatus and the sterilization of the same are often problems of difficult solution. Especially is this true in laboratories where all the media are furnished the students and all the apparatus such as Petri dishes and tubes, etc., are cleaned and sterilized for them. This practice is being followed in the bacteriological laboratories at the University of Illinois.

This paper gives a brief description of apparatus which we have found to be time saving and consequently expense saving in our work. No claim of originality is made. These descriptions are given with the hope that they may be of some use to others who have similar problems to work out.

APPARATUS FOR COOKING MEDIA

This is a double walled steam jacketed trunnion kettle. Similar kettles are used in hotel kitchens and in ice cream plants. Figure 1 shows its general construction and appearance.[1] It is made of one piece of aluminum and hence its inner surface has no seams or joints. It can be used where a supply of steam is available. About 2 to 5 pounds of steam pressure is sufficient for cooking. The kettle used in our laboratory has a capacity of eight gallons.

The main virtue of this kettle lies in the fact that a large quantity of media can be prepared in a short time. It takes less than five minutes to bring 30 quarts of cold water to a boiling point.

[1] This photograph was kindly supplied by the Aluminum Cooking Utensils Company, New Kensington, Pennsylvania.

FIG. 1. STEAM JACKETED TRUNNION KETTLE USED FOR COOKING THE MEDIA

FIG. 2. HOTEL SIZE GAS OVEN USED FOR HOT AIR STERILIZER

APPARATUS FOR STERILIZING WITH HOT AIR

This apparatus is a large gas oven, known as the hotel size oven, because such ovens are used extensively in hotel kitchens. Figure 2 shows its general appearance. The oven is made of non-rustable metal and is constructed on the principle of the fireless cooker, being well insulated and the flame not entering the chambers.

Its main virtues are, first, its size—about 2500 petri dishes can be sterilized at one time; second, ease of sterilization—being well insulated, the oven is heated to a certain temperature and the gas is then turned off; third, the uniformity of sterilization—all parts of the oven are evenly heated. The oven has given great satisfaction.

APPARATUS FOR CLARIFYING MEDIA

Filtration of agar and gelatin has always been a time consuming and unsatisfactory procedure. This is evidenced by the many different methods which have been described in the literature and by the detailed instructions given in the methods for preparing these media. The Laboratory Super-Centrifuge,[2] figure 3, makes it possible to secure clear media without the use of egg albumin and without the trouble of filtering through cotton or paper. With the trunnion kettle and the super-centrifuge, the preparation of a large quantity of media becomes a simple operation. A concrete example may be cited. It took one and one-half hours to prepare, cook, and clarify 25 liters of agar, ready for tubing. With 25 liters of agar there is a loss due to clarifying of not over 500 cc.

These Super-Centrifuge machines may be driven by compressed air or by high pressure steam. Ours is attached to both systems, but we find that the compressed air is more satisfactory for our purpose. A speed of 30,000 revolutions per minute gives a satisfactory filtrate. Equally satisfactory results are secured

[2] This illustration was kindly supplied by the Sharples Company of West Chester, Pennsylvania.

Fig. 3

Fig. 4

Fig. 3. A Cross Section of the Super-Centrifuge
Fig. 4. Test Tube and Flask Washing Machine Driven by Electric Motor

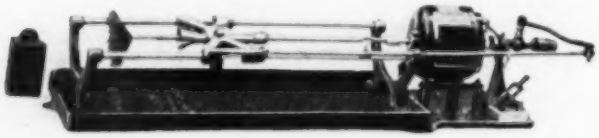

Fig. 5

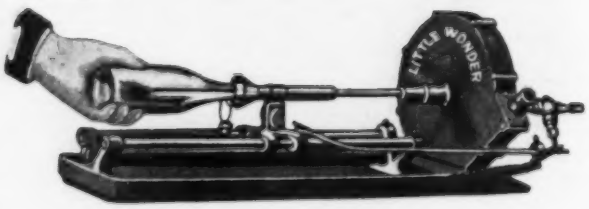

Fig. 6

Fig. 5. Modification of Figure 4
Fig. 6. Test Tube and Flask Washing Machine Driven by Water Motor

with agar as with gelatin. The value of this apparatus is especially noticeable with a stiff agar (2.5 per cent), used in such medium as Endo's agar. With the old methods of clarification the filtration of such stiff agar was a task.

APPARATUS FOR WASHING

The washing of large quantities of test tubes and other apparatus by hand is time consuming and expensive. The high cost of laboratory apparatus makes it imperative that test tubes be cleaned and sterilized as soon as possible in order that they may not be out of use for any extended period of time. We resorted to the use of the contrivance which is used with success in the carbonated drink industry and while it is not directly suited to all bacteriological apparatus, it is serving well its purpose. This apparatus consists of a revolving brush attached to the shaft of an electric motor. Figures 4, 5 and 6 show the general construction of this apparatus. Those in figures 4 and 5 are driven by an electric motor while that in figure 6 is driven by a water motor. Figures 5 and 6 are equipped with a contrivance to admit clean water into the test tube or the bottle while it is being washed by the revolving brush. Any size brush can be attached to the shaft.

In our experience we find that the simple machine shown in figure 4 is as satisfactory as those with the contrivance for admitting water. If these machines are run carefully, breakage of glass ware is a negligible factor with any of them.

MILK-POWDER AGAR FOR THE DETERMINATION OF BACTERIA IN MILK

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Before presenting descriptions of new media for the determination of bacteria in milk, we wish to call attention to the fact that the media described give not only quantitative but, to a certain extent, qualitative results. If the new media achieved merely the same results as those now well known, we should not feel warranted in taking up the reader's time.

It is believed that the time has passed when one can be satisfied with total counts only. Mere quantitative results in bacteriological milk analysis can not be interpreted satisfactorily unless something of the history of the milk is known. Often this "something" is missing; then one must interpret the count as due to this, that, or the other condition or a combination of them. This is not an attempt to discredit the total bacterial count, for it is very valuable as it stands, in milk-control work, but it is hoped that some of the media described in this paper will increase its value.

The media extensively used in milk-control work have been (1) infusion agar, described in the standard methods of the American Public Health Association in 1910, and (2) extract agar, later adopted as a standard medium, its formula having been published by the association in 1916.

Many who use the extract agar realize that it does not give the high counts which are obtained on the infusion agar, but use it because it is the standard medium for milk analysis. Even the members of the Committee on Standard Methods recognized the fact that extract agar is defective, as is shown by the state-

ment on page 5 of the 1916 Standard Methods that "The standard methods, for example, are not such as give a proper count of lactic acid bacteria." In this connection we must say that we see no reason for not taking into account the lactic-acid bacteria. They are of as much significance in tracing the history of a sample of milk as any other type of bacteria, and especially so when their proportion to some other type, such as peptonizers, can be determined. Furthermore, it is believed, since extract agar does not readily support the growth of certain types of lactic-acid bacteria, that this is the most important reason for variations in counts observed when the new standard medium is used. There are certain types of lactic-acid bacteria which will grow slowly on extract agar, and their colonies may be either just visible or not show at all after forty-eight hours' incubation at 37°C.

In our opinion, a medium should be used (1) which will be relatively cheap, (2) which will give the highest count with colonies large enough to be readily counted, and (3) which will give different pictures with different milks; that is, it should be qualitative so far as possible. If bacteria are in milk there is no logical reason for counting only a portion of them. A bacterial count is made to determine the number of bacteria in milk, and if one medium shows higher counts than another, it is evident that the highest count comes closest to the actual number of bacteria in the milk. With either the infusion or extract agar medium nothing but a simple count can be obtained. If a medium can be devised which gives an indication of different groups of bacteria or even only of a single group, in other words, a medium which will give any information relative to the bacteria in milk besides a simple count, it should prove to have distinct advantage over the media now in common use.

GENERAL SCOPE OF THE WORK

In 1911, one of us (Ayers (1911)) described a casein medium which was adapted to the bacterial examination of milk. On this casein medium peptonizers could be readily detected and

counted and the total count obtained as well. It was found that the total counts on the casein medium were decidedly lower than on infusion agar unless the plates were incubated for six days. This fact, of course, made the medium almost valueless for routine control work. Attempts to make a medium by using skimmed milk, with its casein dissolved, and adding peptone and extract were not successful.

Having in mind this casein medium, with its advantages and defects, it seemed probable that skimmed-milk powder could be used in place of purified casein, and that with some additional nutritive substances a cheap and valuable medium could be prepared which, in forty-eight hours, would give counts at least as good as the present standard extract agar and also give a count of the peptonizers present.

In this work about 50 different media were made, containing milk powder alone and in combination with meat extract, yeast extract, peptone, peptone and meat extract, and meat infusion. Ordinary agar was tried, also agar with some of the calcium and magnesium salts removed by precipitation with sodium dibasic phosphate, and agar in which they were partly removed by washing.

The media suggested in this paper are not the result of a "lucky shot." They were developed during a study of various combinations which were in every case compared with the standard extract agar. This work resulted in the examination of about 400 samples of milk of different kinds, that is, raw, pasteurized, and certified; which, figuring the different combinations, is equivalent to the plating of about 1750 samples of milk on a single medium.

This is mentioned only to impress upon the reader that the directions given for making the media are based on considerable experimental data which it is impracticable to include in this paper. We do not feel that the media are beyond improvement, but hope the directions will be followed until they have been given a fair trial.

We will describe a skimmed-milk powder medium containing meat extract and peptone, a modification of this medium, and a milk powder medium containing no meat extract or peptone.

MILK-POWDER AGAR

Medium A. Ingredients for one liter of medium

(a) { 5 grams skimmed-milk powder; 1 gram sodium dibasic phosphate. (Sorensen's phosphate[1]) ($Na_2HPO_4 + 2H_2O$) } In 250 cc. distilled water

(b) { 5 grams peptone; 3 grams extract } In 250 cc. distilled water

Mix (a) and (b) and add 500 cc. of double strength (3 per cent) washed-agar solution.

DETAILED DIRECTIONS FOR PREPARATION OF 1 LITER OF MEDIUM

Milk-powder solution

The detailed directions must be followed accurately if satisfactory and constant results are to be obtained. The medium is very easy to prepare when the various steps are understood and the process completed once. It may appear complicated because of the complete details which are given of each step in the process and which make the preparation of the medium appear somewhat long.

[1] Sorensen's phosphate is sodium dibasic phosphate with 12 molecules of water of crystallization ($Na_2HPO_4 + 12H_2O$) which has been *air dried* so that instead of containing 12 molecules of water it has 2 molecules. Anhydrous sodium dibasic phosphate takes up water easily as it stands in the laboratory and that containing $12H_2O$ loses water easily, so that neither of these can be depended upon to be definite in regard to water content. To overcome this difficulty, Sorensen air-dried $Na_2HPO_4 + 12H_2O$ for about two weeks and found that the water of crystallization was reduced to $2H_2O$ and that it remained practically constant. Sorensen's phosphate can be purchased or for practical purposes prepared in the laboratory by taking the ordinary dibasic phosphate with $12H_2O$ and spreading it out on filter paper and allowing it to remain at room temperature in a dry place for fourteen days. This phosphate ($Na_2HPO_4 + 2H_2O$) is used in the preparation of the medium so that a definite amount can be weighed out at any time and the milk-powder solution will always have practically the same hydrogen-ion concentration. If this phosphate is purchased, care should be taken to be sure it contains two molecules of water and is not anhydrous.

To make the milk-powder solution "*a*" use a good grade of skimmed-milk powder made by the *spray* process, and prepare the following solutions:

(1) 5 grams milk powder
20 cc. distilled water
(2) 1 gram sodium dibasic phosphate (Sorensen's phosphate)
5 cc. distilled water

(*When making more than 1 liter, the same proportions of milk powder to water and phosphate to water must be used; therefore, to make 5 liters multiply each amount by 5.*)

Weigh out 5 grams of skimmed-milk powder and pour on to 20 cc. of *cold* distilled water in a small beaker. Stir until thoroughly dissolved. In another beaker, dissolve 1 gram of sodium dibasic phosphate (Sorensen's phosphate) in 5 cc. of distilled water. Warm to dissolve phosphate quickly. Sorensen's phosphate, $Na_2HPO_4 + 2H_2O$, must be used.

Add the phosphate solution (2) to the milk powder (1). Place the beaker, containing (1) and (2) mixed, in a water bath with water at about 30°C. and heat the milk powder phosphate solution to about 60°C. This should take about 10 minutes. At temperatures between 50 and 60°C. a flocculent grayish precipitate will appear. Continue the heating until the precipitate appears, then steam in an Arnold or other steamer for five minutes, or until the precipitate appears white. Then dilute the milk-powder solution about one-third with distilled water and steam five minutes longer. Too long steaming will cause the solution to turn dark and should be avoided. The whole heating period should not be more than twenty or twenty-five minutes.

Decant the solution while hot on to a filter paper, taking care to keep the precipitate in the beaker until most of the liquid is through. Then pour the precipitate on the filter and wash with a little distilled water. If the milk-powder solution has been properly heated it will filter readily, provided the filter paper is not too hard. "J. Green" Grade 588 and "Ilmenau" filter paper have given good results.

The filtrate, which is of a yellowish-white color, will appear cloudy and can not be filtered clear in the concentration used. This makes no difference, because it is clear in the dilution of the final medium.

Make up the filtered milk-powder solution to 250 cc. with distilled water. This completes the milk-powder solution "a."

PEPTONE-EXTRACT SOLUTION

To make the peptone-extract solution "b" dissolve 5 grams peptone and 3 grams Liebig extract in 100 cc. distilled water by steaming in the Arnold sterilizer or by boiling over flame for twenty minutes. Filter until clear and make solution up to 250 cc. with distilled water. This completes solution "b." "Difco" peptone has been used in our experiments because of its hydrogen-ion concentration, which is near the neutral point, and because, with the extract in the proportion of 5 grams peptone to 3 grams extract, a precipitate is usually formed which permits filtration with a resulting clear solution. For a standard medium, whatever makes of peptone and extract are selected should be universally used.

The milk-powder solution "a" is now mixed with the peptone extract solution "b" which gives a total volume of 500 cc. To this mixture 500 cc. of double strength (3 per cent) washed-agar solution is added. This completes the medium, which is now ready for sterilization.

THE USE OF DISTILLED WATER

We specifically mention distilled water. Tap water may or may not contain dissolved substances, the effect of which on media making and bacterial growth is unknown. By the use of distilled water this uncertainty is obviated and one is assured of a constant definite solvent, "standard" everywhere.

WASHED-AGAR SOLUTION

A stock solution of double strength (3 per cent) washed agar is prepared and put up in flasks and sterilized. This agar is then

ready at any time for use. The agar should be put up in flasks in amounts suitable for the amount of medium to be made at any one time. It is not desirable to use part of a flask of agar, then resterilize and hold for future use. Repeated heating lowers its jelly strength. The flasks should be stoppered to prevent evaporation. To prepare a liter of 3 per cent washed agar, weigh out 30 grams of agar and place in a flask with 2000 cc. of distilled water. This proportion should always be used. Allow it to stand for twenty-four hours, at room temperature, with occasional shaking. Then pour off as much water as possible, using a piece of cheesecloth over the top of the flask, and add distilled water enough to make up again the original volume.

Allow the agar to stand another twenty-four hours, then pour off the agar on to a cotton-flannel cloth in a funnel and wash once with a liter of distilled water. Let the agar drain and then press out as much water as possible by squeezing the filter cloth with the hands. A container large enough to hold the agar is counterpoised on the laboratory scales, and the agar placed in it. In the opposite pan is placed 30 grams for the agar and 1000 grams for the weight of the water in which the agar is to be dissolved. Then water enough is added to make up this weight. This will make a liter of 3 per cent agar. Dissolve the agar by heating in the Arnold sterilizer, then filter through cotton flannel or absorbent cotton until clear.

REASONS FOR USING WASHED AGAR

There are two reasons for using washed agar; first, because it makes possible the preparation of the previously described medium without the formation of a precipitate upon sterilization; and second, because washed agar gives higher counts than ordinary agar, at least with some samples of milk.

When ordinary or so-called purified agars are used in a medium with milk powder, a heavy precipitate forms upon sterilization, probably due to the precipitation of calcium and magnesium phosphate with some casein. This will not occur if enough sodium bicarbonate is added to the medium to bring the hydro-

gen-ion concentration to pH 7.6. The medium with this pH, however, does not give satisfactory results as to counts.

By the addition of $Na_2HPO_4 + 2H_2O$ to the agar during preparation, the calcium and magnesium salts are partly precipitated and can be removed by filtration. This agar when used in the medium causes no precipitation upon sterilization but cuts down the count. From our experiments it is believed this reduction may be due to the increase in the phosphate content of the medium.

Washing the agar as described gives an agar which works perfectly because the calcium and magnesium content is reduced to a considerable extent and a readjustment of the medium to a suitable pH is not necessary.

We will consider the question of the special value of washed agar in raising counts, in another paper.

NO ADJUSTMENT OF REACTION NECESSARY

It will be noted that no adjustment of reaction has been mentioned. If the medium is made according to directions, it will have a hydrogen-ion concentration of about pH 6.8 when "Difco" or Parke-Davis peptone is used and about pH 6.7 with Fairchild peptone. To be safe, it is desirable to check the reaction of the milk-powder peptone-extract solution for its hydrogen-ion concentration with indicators[2] for each new lot of skimmed-milk powder, peptone, and meat extract used. This can easily be determined in a sufficiently accurate manner by adding 5 drops of phenol red indicator to a tube containing 5 cc. of the double-strength milk-powder peptone-extract solution diluted to 10 cc. with distilled water and 5 drops of brom-cresol purple to another tube of the solution. The reaction is correct when the tube with phenol red is yellow and the one with brom-cresol purple is purple. If the tube with phenol red is red, add $\frac{N}{10}$ HCl, calculating the amount necessary to change it to yellow

[2] See paper by Clark, W. M., and Lubs, H. A. The colorimetric determination of hydrogen ion concentration and its application to bacteriology. Journ. Bact., **2**, p. 1, 1917.

with phenol red, and purple with brom-cresol purple. From this computation it is easy to figure the amount of *N* HCl required to bring the total volume of solution to the proper pH. If the color is greenish or yellow with brom-cresol purple add $\frac{N}{10}$ NaOH and calculate the amount of *N* NaOH necessary to bring the total volume of solution to a point where it is purple with brom-cresol purple and yellow with phenol red. This is a very rough way of obtaining a reaction of about pH 6.8 but is accurate enough for this medium. This process need only be carried out, as previously stated, with each new lot of ingredients used. The addition of washed agar does not appreciably affect the reaction.

To prepare the *stock* solution of phenol red indicator take 0.2 gram of the powdered phenol red and add 11.4 cc. of $\frac{N}{10}$ NaOH. Warm and agitate until dissolved, then make up to 100 cc. with distilled water. Take one volume of this stock solution and 9 volumes of distilled water to make the *test* solution. Use 5 drops of this test solution to 10 cc. of medium at 45°C. in a test tube.

The *stock* solution of brom-cresol purple is made by adding 14.8 cc. $\frac{N}{10}$ NaOH to 0.4 gram of powdered brom-cresol purple. This is warmed and when dissolved is made up to 100 cc. with distilled water. To make the *test* indicator solution mix one volume of stock solution with 9 volumes of distilled water. Use 5 drops to a tube with 10 cc. of medium at 45°C. to indicate the hydrogen-ion concentration.

COUNTS OBTAINED WITH THE MEDIUM

Comparisons were made of the counts on standard extract agar, standard infusion agar (1910 standard methods) and milk-powder medium. The plates were incubated at 37°C. for forty-eight hours and a hand lens was used in counting. Various kinds of milk were examined, such as raw, pasteurized, and certified milk. The results of this work are shown in table 1. It will be seen that with three exceptions the counts on milk-powder agar were higher than on the standard extract agar. With the

TABLE 1

Comparison of bacteria counts on extract agar, infusion agar, and milk-powder agar A

EXTRACT AGAR. STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR. STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK POWDER AGAR A.	INCREASE OVER EXTRACT AGAR
Raw milk				
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
650,000	1,080,000	66.0	1,250,000	92.0
1,110,000	1,890,000	70.0	1,760,000	59.0
1,410,000	1,930,000	34.0	1,890,000	34.0
1,160,000	6,110,000	425.0	5,830,000	400.0
1,580,000			4,410,000	180.0
246,000	2,110,000	450.0	687,000	188.0
239,000	1,460,000	510.0	1,500,000	520.0
3,370,000			12,160,000	262.0
1,760,000	2,930,000	66.0	3,500,000	99.0
2,300,000	3,530,000	53.0	4,700,000	100.0
5,120,000			14,720,000	186.0
690,000	860,000	29.0	960,000	39.0
480,000	950,000	100.0	900,000	87.0
59,000	173,000	193.0	215,000	264.0
54,000	220,000	300.0	249,000	366.0
1,100,000	780,000	62.0	2,000,000	82.0
112,000	198,000	76.0	204,000	82.0
217,000	900,000	232.0	970,000	258.0
75,000	510,000	580.0	720,000	860.0
6,800,000	46,200,000	517.0	41,200,000	506.0
2,860,000	6,400,000	124.0	9,800,000	243.0
119,000			447,000	275.0
159,000			256,000	61.0
192,000			284,000	48.0
87,000			124,000	42.0
1,360,000			2,790,000	105.0
124,000			149,000	21.0
1,210,000			1,590,000	31.5
380,000			320,000	−18.0
325,000			380,000	17.0
139,000			303,000	118.0
263,000			391,000	48.5
720,000			1,400,000	94.5
990,000			1,230,000	24.0
130,000			297,000	128.0
154,000			308,000	100.0
141,000			257,000	82.0
620,000			870,000	40.0
196,000			450,000	129.0
184,000			348,000	89.0
167,000			355,000	112.0
9,700,000			16,300,000	68.0
1,200,000			1,580,000	31.0

TABLE 1—*Continued*

EXTRACT AGAR. STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR. STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK POWDER AGAR A.	INCREASE OVER EXTRACT AGAR
Raw milk—*continued*				
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
75,000			105,000	40.0
2,090,000			2,460,000	7.0
60,000			90,000	50.0
4,400,000			6,600,000	50.0
46,000			94,000	104.0
73,000			117,000	60.0
87,000			84,000	−9.0
64,000			85,000	32.0
4,300,000			9,300,000	110.0
43,000			71,000	65.0
127,000			227,000	80.0
227,000			416,000	83.0
163,000			224,000	37.5
145,000			190,000	31.0
2,990,000			3,360,000	20.0
46,000			60,000	30.0
20,000			58,000	190.0
15,700,000			14,500,000	−9.0
4,200,000			5,700,000	35.0
339,000			690,000	104.0
30,300,000			128,000,000	76.0
428,000			740,000	73.0
6,500,000			10,600,000	63.0
48,500,000			153,000,000	202.0
287,000			390,000	36.0
1,420,000			2,200,000	55.0
830,000			1,430,000	72.0
Pasteurized milk				
1,000	68,000	679.0	117,000	11,600.0
3,500	64,000	1,725.0	125,000	3,470.0
41,900	46,000	9.0	62,000	48.0
13,000	20,000	54.0	46,000	254.0
4,800	36,200	652.0	120,000	2,400.0
4,500	25,000	455.0	128,000	2,745.0
5,700	32,000	465.0	140,000	2,350.0
1,500			95,000	6,230.0
3,800	168,000	384.0	191,000	400.0
200	162,000	80,000.0	159,000	75,000.0
6,800	23,400	244.0	16,900	148.0
18,700	49,000	162.0	32,800	75.0
30,000	52,000	73.0	62,000	107.0
51,000	63,000	23.5	111,000	123.0
100,000	112,000	11.0	103,000	3.0

TABLE 1—*Concluded*

EXTRACT AGAR. STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR. STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK POWDER AGAR A.	INCREASE OVER EXTRACT AGAR
Pasteurized milk—*continued*				
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
1,400	36,800	2,500.0	50,000	3,470.0
2,700			7,400	174.0
238,000			288,000	21.0
236,000			313,000	33.0
11,000			55,000	400.0
9,700			56,000	475.0
52,000			154,000	127.0
1,400			61,000	425.0
580,000			2,440,000	320.0
44,000			156,000	255.0
12,500			33,300	166.0
13,200			23,500	78.0
25,000			120,000	41.0
62,000			271,000	330.0
7,700			108,000	1,440.0
980,000			1,240,000	26.0
1,330,000			1,650,000	24.0
66,000			339,000	410.0
220,000			405,000	84.0
300,000			472,000	57.0
47,000			166,000	250.0
36,000			194,000	430.0
24,000			550,000	2,000.0
28,000			370,000	1,220.0
480,000			1,600,000	230.0
600,000			1,040,000	73.0
206,000			470,000	128.0
140,000			760,000	449.0
700			3,700	428.0
300			3,400	1,000.0
6,200			410,000	6,900.0
13,100			560,000	4,200.0
6,400			173,000	2,780.0
16,000			15,500	55.0
9,600,000			17,900,000	65.0
34,800			920,000	2,550.0
Certified milk				
4,900	5,700	16.5	5,200	6.0
8,500	12,400	46.0	14,500	70.0
4,100	7,600	85.0	6,200	51.0
37,800			39,500	4.5
36,000			78,000	110.0
29,100			39,200	25.0

exception of the certified milk the counts were very much higher, the approximate percentage increase over the extract-agar count ranging from 3 to 75,000 per cent. The colonies were very much larger and could be readily counted. Particular care was taken with the count of the colonies on the standard extract agar and we believe our counts are higher than would have been the case if the plates had been counted in the average laboratory, because when there was a question of doubt about a colony it was always included. This was done to avoid favoring the media in which we were interested. The greatest differences in count were usually found in pasteurized milk.

As to the counts on milk-powder agar and infusion agar (1910 standard) it will be noted that of the 36 samples of milk plated, in 22 cases the counts were higher on the milk-powder agar. In some cases they were practically the same or within the limits of error, but in other cases they were distinctly higher.

It seems unnecessary to discuss the results further, for a study of the figures in the table will show clearly the remarkable increase in count obtained on milk-powder agar over that from the standard extract agar.

If the bacteria were not in the milk they would not have been on the milk-powder agar plates; and if one is determining the number of bacteria in a sample of milk one must, if possible, use a medium on which they will grow.

The fact that the medium shows high counts is not its only merit. It is possible with this medium to obtain a direct count of the number of peptonizing bacteria, of strong acid-forming, and of weak acid-forming bacteria in a sample of milk. The medium has been purposely arranged so as to be slightly cloudy after sterilization. After forty-eight hours' incubation, colonies of strong acid-forming bacteria have a white cloud about the colony, while the peptonizers have a clear zone, after the plate has been flowed with acid. Even though the plates are white, due to the precipitation of the dissolved casein through acid formation, it is easy to count the colonies. The colonies of weak acid-forming bacteria can be determined by adding enough brom-cresol purple indicator to cover the plate. This should

remain on the plate for five minutes or until it has penetrated through the agar. All the acid-forming colonies show a yellow color against a purple background. Of course when the plate is crowded the whole plate may be yellow (acid). If desired, brom-cresol purple can be added to the medium at the time of preparation. To get the proper color, add 8.0 cc. of stock solution to each liter of medium; the preparation of the stock solution is described on page 573.

The peptonizers should not be counted until the plate has been flowed with a 5 per cent solution of acetic acid, because the medium is often rendered sufficiently acid to cause a precipitation of the casein except about colonies which produce a strong alkaline reaction. The zone about such colonies under this condition may be clear and resemble peptonization. Flowing with acid will cloud these zones unless there is true peptonization. After flowing the plate with acid allow the acid to stand on the milk-powder medium for ten minutes, then make the count of peptonizers. The acid precipitates the dissolved casein of the milk powder except about colonies where it has been digested. Evidence of this casein peptonization is shown by a clear zone, and colonies should only be counted as peptonizers when there is a definite, clear zone. The activity of the bacteria in the colony can be further estimated by the diameter of the zone. In case no peptonizers show on the plate which is being counted, they should be counted on plates with a lower dilution. They can be easily counted even though the plate is crowded.

When the plate has been flowed with brom-cresol purple, then with acetic acid, the plate is yellow instead of white. This does not interfere in any way with the counting of the peptonizers.

The milk-powder medium, therefore, gives a picture which varies greatly with different samples of milk. Sometimes there are numerous colonies of strong acid-forming bacteria, sometimes none, sometimes many peptonizers and again only a few. While the relation between these various colonies and the total count has not been fully worked out, we believe that there are certain correlations which will be found to have a definite relation to the history of the milk.

To take the fullest advantage of the medium the counting procedure should be as follows:

1. *Make a total count.*
2. *Count strong acid-forming colonies.* Those with a cloudy zone about them or a slight hazy edge.
3. *Count weak acid-forming colonies.* This is done by flowing the plate with brom-cresol purple and counting the total acid colonies and subtracting the number of strong acid-forming colonies.
4. *Count peptonizers.* Flow plate with a 5 per cent solution of acetic acid before counting.
5. *Calculate the number of alkali formers and inert colonies.* To do this add the total number of acid colonies to the number of peptonizers and subtract from the total count.

MILK-POWDER MEDIUM "B"

Feeling that there might be some objection to the use of a medium which was slightly cloudy, it was decided to work out a medium which would be clear. This milk-powder medium differs from the first merely in the amounts of peptone and meat extract used. To make this medium, simply substitute in the formula for medium "A" the following: 2 grams of peptone and 1 gram meat extract instead of 5 grams peptone and 3 grams extract. The medium in other respects is made exactly the same. No adjustment of reaction is necessary, as it will be about pH 7.0 to pH 7.1, brown or slightly red brown with phenol red.

The counts obtained on this medium and other media are shown in table 2. It will be seen that they are just as high on the whole as on milk-powder medium "A" and in many cases higher. The colonies of strong acid-forming bacteria can be seen on this medium with a small white zone about them. They are not so distinct, however, as on the milk-powder medium "A." In order to distinguish colonies of peptonizing bacteria it is necessary to flow the plate with 5 per cent acetic acid. This precipitates the casein and causes the clear medium to have a white, opaque appearance. After flowing with acid, clear zones may be seen about the colonies of the peptonizers.

TABLE 2

Bacterial counts on milk-powder agar A and B compared with extract agar and infusion agar

EXTRACT AGAR, STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR, STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK-POWDER AGAR B (0.2 PER CENT PEPTONE), (0.1 PER CENT MEAT EXTRACT)	INCREASE OVER EXTRACT AGAR	MILK-POWDER AGAR A, (0.5 PER CENT PEPTONE), (0.3 PER CENT MEAT EXTRACT)	INCREASE OVER EXTRACT AGAR
Raw milk						
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
690,000	860,000	29.0	990,000	42	960,000	39
480,000	950,000	100.0	1,090,000	112	900,000	87
59,000	173,000	193.0	180,000	200	215,000	204
54,000	220,000	300.0	229,000	324	249,000	360
1,100,000	1,780,000	62.0	2,600,000	136	2,000,000	82
112,000	198,000	76.8	213,000	90	204,000	82
271,000	900,000	232.0	840,000	210	970,000	258
75,000	510,000	580.0	570,000	660	720,000	860
6,800,000	46,200,000	517.0	44,200,000	550	41,200,000	500
2,860,000	6,400,000	124.0	10,200,000	257	9,800,000	243
Pasteurized milk						
3,800	168,000	384.0	196,000	415	191,000	400
200	162,000	80,000.0	192,000	90,000	159,000	75,000
6,800	23,400	244.0	23,900	250	16,900	148
18,700	49,000	162.0	32,300	73	32,800	75
30,000	52,000	73.0	86,000	187	62,000	107
51,000	63,000	23.5	77,000	51	111,000	123
100,000	112,000	11.0	134,000	13	103,000	3
1,400	36,800	2,500.0	70,000	4,900	50,000	3,470
Certified milk						
4,100	7,600	85.0	6,500	58	6,200	51
4,900	5,700	16.3	6,500	32	5,200	6

This milk-powder agar "B" does not give quite such characteristic pictures with different samples of milk as does the first mentioned milk-powder agar "A." However, it is a clear medium and is cheaper than medium "A."

MILK-POWDER YEAST AGAR

It is sometimes said that it is impossible to expect uniformity in bacteria counts as long as peptone and meat extract are so variable. Of course every one will agree that different makes

of peptone will not be exactly the same and the peptone of one manufacturer may vary from time to time. Even meat extract is not constant in composition. In view of these facts it may be well to eliminate peptone and meat extract from standard media and so discount any possible effect of variations in their composition. With this thought in mind a medium was devised consisting of skimmed-milk powder, yeast extract and washed agar. Yeast extracts (Ayers and Rupp, 1920) have been used extensively in these laboratories and found to be very satisfactory for many purposes.

Ingredients for 1 liter of medium

(a) { 5 grams skimmed-milk powder; 1 gram sodium dibasic phosphate. (Sorensen's phosphate) } In 250 cc. distilled water

(b) 10 grams pure dry fresh yeast. In 250 cc. distilled water

Mix (a) and (b) and add 500 cc. of double strength (3 per cent) washed-agar solution.

DIRECTIONS FOR PREPARATION OF MEDIUM

The milk-powder solution should be prepared as previously described.

The yeast extract is made from dry fresh (not autolyzed) yeast which contains no added fillers. To facilitate filtering, the yeast should be dried at a temperature of from 105° to 110°C. for five hours. The extract is made by steaming 10 grams of yeast in 100 cc. distilled water for 45 minutes in the Arnold, then filtering until the filtrate is clear and brilliant. When most of the extract has been filtered, 100 cc. of distilled water should be poured on the filter to wash out the remainder. Make up to 250 cc. with distilled water. The filter papers previously mentioned for filtering the milk-powder solution give good results with yeast extracts. In order to obtain a clear, brilliant filtrate, it is usually necessary to pour the filtrate back into the filter a few times until it clogs slightly.

The milk-powder solution and yeast extract are then mixed and 500 cc. of double strength (3 per cent) *washed* agar is added.

The washed-agar solution is prepared as described. No adjustment of reaction is necessary, as the final hydrogen-ion concentration of the medium will be about pH 6.7.

BACTERIAL COUNTS ON MILK-POWDER YEAST MEDIUM

The counts obtained on the milk-powder yeast agar, as well as on standard extract and standard infusion agar, are shown in table 3. The figures show that the counts on the milk-powder yeast were much higher than on standard extract agar and that they agree closely with the counts on standard infusion agar.

Yeast extract was used extensively in other combinations with good results, and while we have only a few results on the medium just described it is believed it can be used to advantage with skimmed-milk powder as a substitute for peptone and meat extract. Further work is necessary before this point can be definitely settled.

The milk-powder yeast agar does not appear to give quite such high counts with all samples as the powder, peptone, meat-extract agars A and B, but it has the advantage of being more nearly uniform in composition. This is obvious for the reason that the yeast extract can be made in the laboratory in a definite way from a dry preparation consisting of a definite species of pure yeast.

The milk-powder yeast agar is a clear medium on which colonies of the strong acid-forming bacteria can be seen, although not so distinctly as on the other media described in this paper. Peptonizers can be readily observed by flowing the plate with 5 per cent acetic acid.

THE USE OF SKIMMED-MILK POWDER

Dissolved skimmed-milk powder is used as a foundation for the media described in this paper and not as merely something to be added to some other medium. The use of skimmed-milk powder must be considered in this light in order that its value may be fully appreciated.

TABLE 3

Comparison of bacteria counts on extract agar, infusion agar, and milk-powder yeast agar

EXTRACT AGAR, STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR, STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK-POWDER YEAST AGAR	INCREASE OVER EXTRACT AGAR
Raw milk				
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
690,000	860,000	29.0	810,000	17.0
480,000	950,000	100.0	730,000	52.0
59,000	173,000	193.0	165,000	180.0
54,000	220,000	300.0	209,000	287.0
1,100,000	1,780,000	62.0	1,530,000	39.0
112,000	198,000	76.8	185,000	65.0
271,000	900,000	232.0	660,000	143.0
75,000	510,000	580.0	690,000	820.0
6,800,000	46,200,000	517.0	41,600,000	497.0
2,800,000	6,400,000	124.0	5,800,000	103.0
43,000			80,000	86.0
127,000			198,000	56.0
227,000			342,000	39.0
163,000			200,000	36.0
145,000			158,000	8.0
2,990,000			3,370,000	13.0
46,000			62,000	34.0
20,000			60,000	200.0
Pasteurized milk				
3,800	168,000	384.0	202,000	432.0
200	162,000	80,000.0	192,000	90,000.0
6,800	23,400	244.0	23,400	244.0
18,700	49,000	162.0	32,200	73.0
30,000	52,000	73.0	76,000	153.0
51,000	63,000	25.5	66,000	29.5
100,000	112,000	11.0	107,000	7.0
85,000			132,000	67.0
62,000			291,000	370.0
7,700			114,000	1,530.0
980,000			1,190,000	22.0
1,330,000			1,400,000	5.0
66,000			317,000	380.0
220,000			265,000	21.0
300,000			302,000	0.6
47,000			289,000	510.0
36,000			163,000	353.0
24,000			330,000	1,260.0

TABLE—3 *Continued*

EXTRACT AGAR, STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR, STANDARD METHODS A. P. H. A. 1910	INCREASE OVER EXTRACT AGAR	MILK-POWDER YEAST AGAR	INCREASE OVER EXTRACT AGAR
Pasteurized milk—*continued*				
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
28,000			560,000	1,900.0
480,000			2,000,000	325.0
600,000			1,410,000	135.0
206,000			450,000	118.0
140,000			510,000	264.0
700			2,400	243.0
300			2,300	667.0
Certified milk				
4,100	7,600	85.0	6,800	66.0
4,900	5,700	16.3	5,900	20.0
36,000			69,000	92.0
29,100			37,900	32.0

A medium with skimmed-milk powder and washed agar without any other nutritive material gives counts much higher than the standard extract agar. As may be seen in table 4, the counts were much higher except in the case of the last sample. In many samples the counts on the milk-powder agar, with no other nutritive ingredients, checked with those on standard infusion agar, while with a few samples the count was decidedly lower. The colonies, however, on this medium are very small in most cases; therefore, it is necessary to supply some other nitrogenous material to stimulate growth.

Peptone and meat or yeast extract, whichever may be used, are added in order to increase the nutritive value of the medium. Milk powder supplies lactose to the medium in small amounts, which assists materially in stimulating and supporting bacterial growth, as has been shown by the work of Sherman (1916) and others. We do not feel, however, that the lactose is the only reason for the high counts obtained on media containing dissolved milk powder.

The increased count over extract agar and the large size of the colonies obtained on the media described in this paper are

due, we believe, to a combination of the following factors: the nitrogenous portions of the milk powder, the lactose, the buffer content, and the use of *washed* agar.

When 5 grams of skimmed-milk powder per liter of medium is used, figuring on an average analysis of 50 samples of milk powder, there is added about 0.25 per cent lactose and 0.17 per

TABLE 4

The value of milk powder as a nutrient material

EXTRACT AGAR, STANDARD METHODS A. P. H. A. 1916	INFUSION AGAR, STANDARD METHODS A. P. H. A. 1916	INCREASE OVER EXTRACT AGAR	MILK-POWDER AND WASHED AGAR. NO OTHER INGREDIENTS	INCREASE OVER EXTRACT AGAR
Raw milk				
bacteria per cc.	*bacteria per cc.*	*per cent*	*bacteria per cc.*	*per cent*
690,000	860,000	29.0	770,000	11.5
480,000	950,000	100.0	840,000	75.0
59,000	173,000	193.0	164,000	178.0
54,000	220,000	300.0	162,000	200.0
1,100,000	1,780,000	62.0	1,670,000	50.0
112,000	198,000	76.8	176,000	57.0
271,000	900,000	232.0	810,000	199.0
6,800,000	46,200,000	517.0	36,600,000	395.0
2,860,000	6,400,000	124.0	4,800,000	68.0
Pasteurized milk				
3,800	168,000	384.0	101,000	165.0
200	162,000	80,000.0	186,000	85,000.0
6,800	23,400	244.0	24,100	255.0
18,700	49,000	162.0	29,700	62.0
30,000	52,000	73.0	60,000	100.0
51,000	63,000	21.5	68,000	33.4
100,000	112,000	11.0	114,000	11.0
1,400	36,800	2,500.0	36,000	2,510.0
4,100	7,600	85.0	5,500	34.0
4,900	5,700	16.3	2,000	−59.0

cent protein (mostly casein). From results of the analysis of skimmed-milk powder which were kindly supplied to us by Dr. J. T. Kiester, of the Bureau of Chemistry, United States Department of Agriculture, we have calculated the difference in percentage of lactose and protein if 5 grams of powder containing the highest and lowest percentages found among 20 samples had been used.

The range in the percentage of lactose in the medium would be only 0.03 per cent and in protein also 0.03 per cent. These differences are so slight that they would exert no influence on the medium. It is realized that there may be other differences in skimmed-milk powder, small differences which may not be apparent from the ordinary chemical analysis, and which may be ascribed to the condition of the milk before it was dried, or perhaps to methods of drying.

We have obtained the best results when using a skimmed-milk powder made by the spray process, and suggest that if milk powder is used in a standard medium a spray-process powder be specified, and one prepared by some one company, perhaps even from some special milk.

THE ESTIMATED COST OF DIFFERENT AGAR MEDIA

We are not prepared to say just how important the cost of a medium is, but it will always be a factor and therefore must be considered.

The cost of different agar media has been calculated on a basis of the cost of ingredients shown in the list.

Cost of ingredients

Peptone	$4.80 for 500 grams
Meat extract	3.70 for 1 pound
Dry yeast	3.75 for 500 grams
$Na_2HPO_4 + 2H_2O$	1.50 per pound
Skimmed-milk powder	.50 per pound
Lean beef	.35 per pound
Agar (shred)	.90 per pound

Approximate cost of agar media

	per liter
Standard infusion agar about	$0.46½
Standard extract agar about	.10
Milk-powder agar "A"	.10¾
Milk-powder agar "B"	.06¼
Milk-powder yeast agar	.11½

In making calculations we have not figured the cost of washing agar. This should not add much to the cost. From these figures it may be seen that the milk-powder media cost about

the same as standard extract agar, with the exception of medium B, which costs only about 6 cents a liter. It seems evident that no objections can be raised to milk-powder media on the ground of cost.

SUMMARY

1. Formulae for three agar media are given, two of these media containing skimmed-milk powder with different amounts of peptone and meat extract. The third contains skimmed-milk powder and yeast extract, no peptone or meat extract being used. This may be a distinct advantage if the variation in composition of peptone and meat extract plays an important part in connection with the bacteria count.

2. Any of the skimmed-milk powder media described give counts very much higher than standard extract agar and the colonies are very much larger. The larger size of the colonies makes the counting process much more accurate. Counts obtained on the powder media with peptone and extract are on the average higher than those obtained on the old standard infusion. On milk-powder yeast agar they may be, in general, slightly lower.

3. With these milk-powder media it is possible to obtain not only a total count, but a count of colonies of strong and weak acid-producing, alkali-forming, inert and peptonizing bacteria. The plates therefore, give quantitative and qualitative results, at least so far as these groups of bacteria are concerned.

4. The importance of using washed agar in media for bacterial counts will be discussed further in another paper.

5. It is hoped that these media will be given a thorough trial, for it is felt that any one of the three possesses decided advantages over the present standard extract agar.

No claim is made that the skimmed-milk powder media can not be improved; we hope they can. As they stand at present, however, any one of the three has advantages enough over the extract agar to replace it as a standard medium for the determination of bacteria in milk, because: first, they show higher counts than the standard extract agar and therefore counts

more closely related to the actual number of bacteria in the milk; secondly, they give different "pictures" when different samples of milk are plated; and, thirdly, this is accomplished without any significant increase in expense. In fact, one of these media is much cheaper than extract agar. All the milk-powder media are very much cheaper than the old standard infusion agar.

6. The full value of these milk-powder agar media can be determined only by the results obtained from the analysis of a large number of samples of milk. While our experiments are extensive, we realize that they only indicate the probable value of the media.

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THE USE OF WASHED AGAR IN CULTURE MEDIA

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In connection with the preparation of milk-powder agar it was found that it was necessary to wash the agar in order that it might be used in the medium without causing a precipitate during sterilization. Qualitative tests indicated that the calcium and magnesium salts in the agar probably combined with the phosphate used in the preparation of the milk-powder solution and the phosphates were precipitated during sterilization. To overcome this difficulty, the agar was washed in distilled water in order to reduce its salt content.

Soil bacteriologists have employed washed agar for many years. It has been washed in order to remove as much as possible of the soluble organic matter which is believed to be detrimental to the growth of nitrifying bacteria. Sternberg in his "Textbook of Bacteriology" recommended that agar be soaked in cold water for twenty-four hours; this was advised in order to facilitate dissolving and filtering. It is the practice in some laboratories to wash agar, apparently more for the sake of a cleaner preparation than for any other reason.

Washed agar gave such satisfaetory results in the milk-powder medium that its use in the regular standard peptone extract medium was tried. It is the purpose of this paper to present briefly the results of this work and to show the effect of washing on the calcium and magnesium content of agar.

The results in table 1 show in a decided manner the effect on the bacterial count of using washed agar. While there was not an increase in all samples, there was in many cases an increase beyond any experimental error. As a rule, the higher counts were found when pasteurized milk was examined.

TABLE 1

Bacteria counts on standard extract agar using shred and washed agar

STANDARD EXTRACT AGAR	STANDARD INGREDIENTS AND WASHED AGAR	APPROXIMATE INCREASE OVER STANDARD EXTRACT AGAR
	Raw milk	
bacteria per cc.	*bacteria per cc.*	*per cent*
650,000	990,000	52.0
1,110,000	1,530,000	37.5
1,410,000	1,940,000	37.5
1,160,000	5,180,000	345.0
1,580,000	3,620,000	129.0
246,000	432,000	75.0
239,000	1,010,000	320.0
3,370,000	11,580,000	246.0
1,760,000	3,040,000	72.6
2,300,000	4,330,000	88.2
5,120,000	15,360,000	199.0
139,000	198,000	42.5
263,000	346,000	31.5
720,000	1,010,000	40.0
990,000	800,000	−23.0
130,000	224,000	72.0
154,000	259,000	68.2
141,000	144,000	2.1
620,000	790,000	27.0
196,000	232,000	18.4
184,000	230,000	25.0
167,000	189,000	13.2
9,700,000	16,800,000	73.0
30,300,000	50,400,000	66.0
15,700,000	3,200,000	−20.0
4,200,000	3,500,000	−8.0
339,000	264,000	−7.8
428,000	189,000	−44.0
6,500,000	6,100,000	−9.5
48,500,000	36,300,000	−7.5
287,000	228,000	−8.0
1,420,000	1,050,000	−7.0
830,000	740,000	−9.0
	Pasteurized milk	
1,600	2,100	31.0
1,000	8,000	700.0
3,500	17,000	385.0
41,900	63,000	50.0

TABLE 1—*Continued*

STANDARD EXTRACT AGAR	STANDARD INGREDIENTS AND WASHED AGAR	INCREASE OVER STANDARD EXTRACT AGAR
Pasteurized milk—*continued*		
bacteria per cc.	*bacteria per cc.*	*per cent*
13,000	22,000	69.0
4,800	66,000	1275.0
45,000	76,000	69.0
5,700	68,000	1090.0
1,500	20,100	1240.0
52,000	84,000	61.5
1,400	3,100	121.0
580,000	870,000	50.0
44,000	94,000	135.0
12,500	18,600	48.0
13,200	19,700	48.0
62,000	88,000	42.0
13,100	15,700	19.0
6,400	37,000	480.0
10,000	14,900	49.0
9,600,000	11,520,000	19.0
34,800	71,000	104.0

The washed agar used in these experiments was prepared as follows: For 1 liter of double strength (3 per cent) agar, 30 grams of shred agar was placed in a flask with 2000 cc. of distilled water. This was allowed to stand for 24 hours at room temperature. At the end of this period as much water as possible was poured off, a piece of cheesecloth being placed over the top of the flask. Fresh distilled water was added to make up for the water poured off. The agar was allowed to soak another twenty-four hours, after which it was thrown on to a cotton-flannel cloth in a funnel and washed once with 1 liter of distilled water. The agar was allowed to drain and as much as possible of the remaining water was pressed out by squeezing the filter cloth with the hands. A container large enough to hold the agar was counterpoised on the laboratory scales, and the agar was placed in it. In the opposite pan was placed 30 grams for the weight of the agar and 1000 grams for the weight of the water in which it was to be dissolved. Then water enough was added to the agar to make up this weight, it was then dissolved and filtered.

After the agar is washed, it may be air dried, and then used the same as ordinary agar. It is only necessary to use 1 per cent of the dry material to obtain a medium having the same jelly strength as a 1.5 per cent shred agar medium.

Various methods were tried for washing shred agar, and the effect of these treatments on the calcium and magnesium content will be of interest. As shown in table 2, ordinary shred agar contained about 16 per cent of moisture. On an air-dry basis

TABLE 2

Analyses of various kinds of agar

KIND OF AGAR	MOISTURE	ON DRY BASIS			
		Ash	CaO	MgO	Protein
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Shred agar					
Sample 1	16.37	4.46	1.17	0.79	2.29
Sample 2	15.97	4.48	1.13	0.76	2.23
Commercially purified agar					
Lot No. 1	12.83	4.54	1.41	0.64	1.66
Lot No. 2	8.82	3.77	1.12	0.55	1.40
Washed agar					
24 hours (A)	14.21	3.37	0.74	0.53	
48 hours (B)	14.62	2.99	0.70	0.39	
72 hours (C)	15.45	2.74	0.61	0.34	
24 hour washed 30 grams to 5000 H_2O	14.32	2.74	0.61	0.43	
NaCl-acid treated agar	12.70	3.37	0.39	0.08	1.35

the per cent of ash was about 4.4. The calcium content was about 1.15 per cent, calculated as CaO, and the magnesium about 0.77 per cent, calculated as MgO.

Two lots of commercially purified agar were examined. These were purchased at different times, and probably do not represent one orginal lot of the material. The agar was said to contain a minimum amount of moisture, when packed, and to have been specially treated to reduce the extraneous matter, inorganic

salts, and acidity. As the results show, the moisture content of this agar was variable, but the content of calcium was no lower than that of shred agar, although the magnesium had been slightly reduced. The protein content, however, was about 50 per cent of that of shred agar.

The analysis of washed agar showed a decided decrease in the percentage of calcium and magnesium, and experiments were made to determine the time required to wash the agar, starting with 30 grams to 2000 cc. of water. Agar "A" was held twenty-four hours. Agar "B" was held forty-eight hours, but after the first twenty-four hours the 2 liters of water were replaced with fresh water. Agar "C" was held seventy-two hours, the water being removed and replaced with fresh distilled water at the end of each twenty-four hours. The agar was air dried by spreading it on filter papers after washing.

It will be seen from the table that the moisture content of the three lots of agar was very similar to that of shred agar. The most interesting effect of washing was the reduction of the calcium and magnesium salts. The forty-eight-hour period of washing, which is the one we have used most extensively, reduced the CaO from about 1.1 per cent to 0.7 per cent, and the MgO from about 0.78 per cent to 0.39 per cent. The calcium and magnesium content was reduced sufficiently by this treatment to give satisfactory results in the milk-powder agar medium; that is, enough was removed so that the phosphates did not precipitate upon sterilization. Agar washed twenty-four hours in the proportion of 30 grams to 2000 cc. of water was not entirely satisfactory and, as the results show, there was but little further reduction in the calcium and magnesium by washing for seventy-two hours.

It was found that by increasing the amount of distilled water, good results could be obtained in twenty-four hours. When 30 grams of shred agar was held in 5000 cc. of distilled water for twenty-four hours the calcium and magnesium content was found to be 0.61 per cent and 0.43 per cent respectively, when calculated on a moisture-free basis.

After the agar is washed, it may be air dried, and then used the same as ordinary agar. It is only necessary to use 1 per cent of the dry material to obtain a medium having the same jelly strength as a 1.5 per cent shred agar medium.

Various methods were tried for washing shred agar, and the effect of these treatments on the calcium and magnesium content will be of interest. As shown in table 2, ordinary shred agar contained about 16 per cent of moisture. On an air-dry basis

TABLE 2

Analyses of various kinds of agar

KIND OF AGAR	MOISTURE	ON DRY BASIS			
		Ash	CaO	MgO	Protein
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Shred agar					
Sample 1	16.37	4.46	1.17	0.79	2.29
Sample 2	15.97	4.48	1.13	0.76	2.23
Commercially purified agar					
Lot No. 1	12.83	4.54	1.41	0.64	1.66
Lot No. 2	8.82	3.77	1.12	0.55	1.40
Washed agar					
24 hours (A)	14.21	3.37	0.74	0.53	
48 hours (B)	14.62	2.99	0.70	0.39	
72 hours (C)	15.45	2.74	0.61	0.34	
24 hour washed 30 grams to 5000 H_2O	14.32	2.74	0.61	0.43	
NaCl-acid treated agar	12.70	3.37	0.39	0.08	1.35

the per cent of ash was about 4.4. The calcium content was about 1.15 per cent, calculated as CaO, and the magnesium about 0.77 per cent, calculated as MgO.

Two lots of commercially purified agar were examined. These were purchased at different times, and probably do not represent one orginal lot of the material. The agar was said to contain a minimum amount of moisture, when packed, and to have been specially treated to reduce the extraneous matter, inorganic

salts, and acidity. As the results show, the moisture content of this agar was variable, but the content of calcium was no lower than that of shred agar, although the magnesium had been slightly reduced. The protein content, however, was about 50 per cent of that of shred agar.

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It will be seen from the table that the moisture content of the three lots of agar was very similar to that of shred agar. The most interesting effect of washing was the reduction of the calcium and magnesium salts. The forty-eight-hour period of washing, which is the one we have used most extensively, reduced the CaO from about 1.1 per cent to 0.7 per cent, and the MgO from about 0.78 per cent to 0.39 per cent. The calcium and magnesium content was reduced sufficiently by this treatment to give satisfactory results in the milk-powder agar medium; that is, enough was removed so that the phosphates did not precipitate upon sterilization. Agar washed twenty-four hours in the proportion of 30 grams to 2000 cc. of water was not entirely satisfactory and, as the results show, there was but little further reduction in the calcium and magnesium by washing for seventy-two hours.

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The results obtained by the addition of NaCl and HCl to the wash water are interesting. Various amounts of salt and acid were used, their use having been suggested by Dr. Zoller of these laboratories. The best results were obtained as follows: 20 grams of shred agar was added to 1000 cc. of distilled water containing 10 grams of NaCl and 5 cc. of N/10 HCl. This was allowed to stand for six hours at room temperature, then the salt and acid solution was poured off and replaced by 1800 cc. of fresh distilled water. This was allowed to stand 18 hours, which made the washing period twenty-four hours. The agar was then poured on a cotton-flannel cloth in a funnel and washed with 500 cc. of distilled water, then allowed to drain and as much water pressed out by hand as possible. The agar was finally air dried.

The agar treated in this manner showed a lower calcium and magnesium content than any of the others. Reference to the table shows that it contained 0.39 per cent of CaO, and 0.08 per cent MgO, while the protein content was reduced from about 2.2 per cent to 1.35 per cent.

Media made with this agar did not, however, give quite such satisfactory counts as that made with washed agar, but its use has not been tried out very extensively. This method of washing, however, has possibilities of considerable value. The principal point of interest at present in connection with the use of NaCl and HCl is the fact that they assist in the removal of the calcium and magnesium salts.

We were very much interested in the results obtained with washed agar. It was valuable to us because it made possible a milk-powder agar medium which would stand sterilization without precipitation of the phosphates, but the fact that higher bacterial counts were often obtained when it replaced ordinary shred agar in the standard extract medium led us to wonder just what might be the explanation.

In connection with the milk-powder medium it had been noticed that the presence of too much phosphate often lowered the bacterial counts. Even the increase from 0.1 per cent to 0.2 per cent seemed to have a marked effect. Besides this, in

certain milk-powder media the milk powder was dissolved in such a way as to leave the calcium phosphate in the medium. With such media low counts were noticed.

Having these facts in mind and knowing that at least one of the effects of washing agar was a partial removal of the calcium and magnesium salts it occurred to us that this lowering of the percentage of these salts might be one of the reasons for the increased counts when washed agar was used in the standard medium. To confirm this opinion, counts were made on three media, all of which contained 0.5 per cent peptone and 0.3 per cent extract, and had the same reaction but made up with different kinds of agar. One contained regular shred agar, one washed agar, and the other washed agar with sufficient $CaSO_4$ and $MgSO_4$ added to make up for the calcium and magnesium (calculated as CaO and MgO) removed by washing.

TABLE 3

Effect of addition of calcium and magnesium salts to a washed-agar medium

STANDARD EXTRACT AGAR, A. P. H. A. 1916	STANDARD EXTRACT WASHED-AGAR AND Ca AND Mg SALTS	STANDARD EXTRACT WASHED AGAR
bacteria per cc.	*bacteria per cc.*	*bacteria per cc.*
6,200	5,800	8,800
13,100	8,100	15,700
6,400	6,700	37,000
10,000	12,000	14,900
9,600,000	9,550,000	11,500,000
34,800	30,600	71,000

A few samples of pasteurized milk were plated on these three media. The results in table 3 indicate that the addition of these salts to the washed-agar medium reduced the counts so that they agreed with those on the regular agar medium.

We do not intend to convey the idea that this is an explanation of the higher counts. Perhaps similar results may never be obtained again, but at least they indicate that these salts may play an important part in culture media.

SUMMARY AND CONCLUSIONS

1. The standard extract medium with washed agar showed in many cases, when market milk was examined, a much higher count than the same medium with regular shred agar.

2. Washing agar reduced its content of calcium and magnesium salts.

3. A few experiments indicated that the removal of these salts was a factor in the cause of the higher counts. This point, however, is merely suggested by the results and not definitely proved.

4. Since certain samples of milk show a higher count when plated on a washed-agar standard extract medium than on the regular standard extract medium, it seems evident that washing removes something detrimental to the growth of certain species of bacteria. This naturally suggests that a further study is needed of the value of washed agar in lines of bacteriological work where it has not been used.

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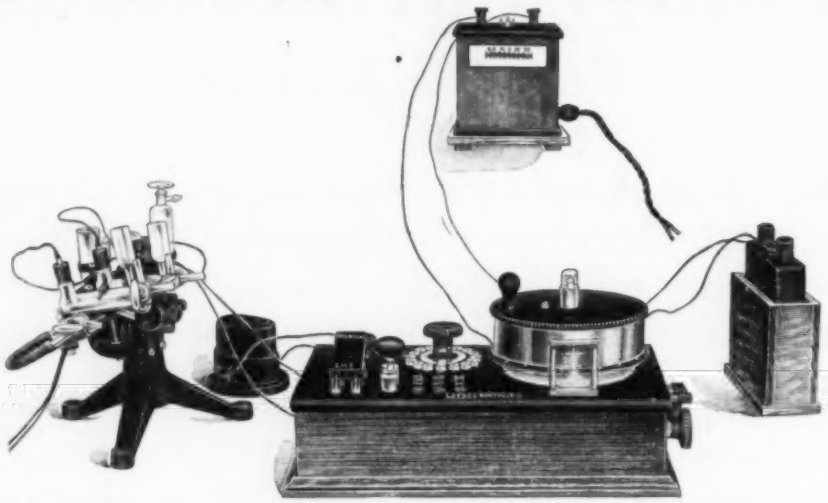

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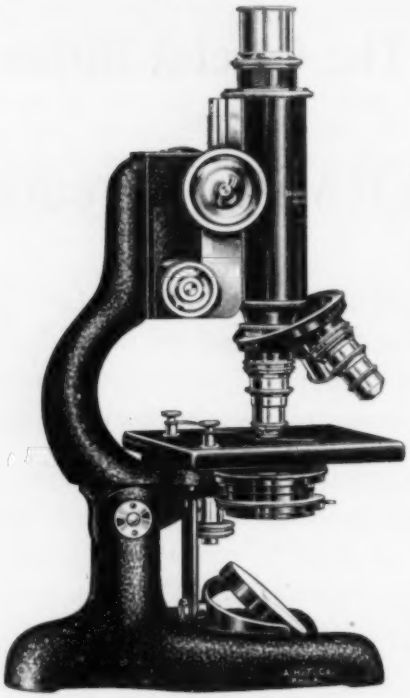

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